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(54) Title: IMPROVED PLASTID TRANSFORMATION OF HIGHER PLANTS AND PRODUCTION OF TRANSGENIC PLANTS WITH HERBICIDE RESISTANCE

(57) Abstract

The present invention provides a method of producing a herbicide–resistant plant, which method entails delivering one or more herbicide resistance–conferring selectable marker genes into and expressing the same within the plastid of the plant, both in photosynthetic as well as non–photosynthetic cells. Nucleic acids for transformation and multicellular plants whose plastids have been transformed are also provided.

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IMPROVED PLASTID TRANSFORMATION OF HIGHER PLANTS AND PRODUCTION OF TRANSGENIC PLANTS WITH HERBICIDE RESISTANCE

This is a continuation-in-part of U.S. Application Serial No. 08/899,061, filed July 23, 1997.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to the genetic engineering of plant plastids, particularly plastids of non-photosynthetic cells. The invention provides selectable marker genes and genetic constructs for the expression of foreign genes in the plastids of higher plant species. The invention also provides a novel approach to creating herbicide resistance in transgenic plants and transgenic plants thereby produced.

Background of the Invention

Extensive work has been carried out during the past decade in the development of glyphosate-resistant plants (for review, see G. Barry *et al.* in Biosynthesis and Molecular Regulation of Amino Acids in Plants., B.K. Singh *et al.* [ed.], Am. Soc. Plant Physiologists, Rockville, MD (1992)). One of the many attractive features of this non-selective herbicide is its rapid degradation by soil microorganisms. Glyphosate specifically binds to and blocks the activity of 5-enolpyruvylshikimate-3-phosphate synthase (EPSP synthase, EPSPS) (E.C. 2.5.1.19) (Steinrucken and Amrhein, *Biochem. Biophys. Res. Comm.* **94**, 1207 (1980)), an enzyme of the aromatic amino biosynthesis pathway. EPSPS catalyzes the reaction of shikimate-3-phosphate and phosphoenolpyruvate (PEP) to form 5-enolpyruvylshikimate-3-phosphate (EPSP) and phosphate. Glyphosate is a competitive inhibitor of EPSPS with respect to PEP, and prevents the synthesis of aromatic amino acids essential for the synthesis of protein and

certain secondary metabolites. Importantly, EPSPS activity is plastid-localized; it is a nuclear-encoded protein that is synthesized in the cytosol and then imported into the plastid, the site of aromatic amino acid biosynthesis (della-Cioppa *et al.*, *Proc. Natl. Acad. Sci. USA* **83**, 6873 (1986) ("della-Cioppa I").

The *E. coli hph* gene has been widely utilized as a selectable marker gene for the recovery of hygromycin B-resistant nuclear transformants, both microbial and plant (Gritz and Davies, *supra*; C. Waldron *et al.*, Plant Mol. Biol., 5, 103 (1985)). The *hph* gene product, hygromycin phosphotransferase (HPH), confers resistance by phosphorylation of the antibiotic, hygromycin B. Peñaloza-Vazquez *et. al.* (*Appl. Environ. Microbiol.* **61**, 538 (1995) ("Peñaloza-Vazquez I")) demonstrated that glyphosate was also utilized as a substrate for phosphorylation by hygromycin phosphotransferase, thus permitting the growth of glyphosate-resistant *E. coli* and tobacco plants. Peñaloza-Vazquez and co-workers (Peñaloza-Vazquez *et al.*, *Plant Cell Rep.* **14**, 482 (1995) ("Peñaloza-Vazquez II")) expressed the *E. coli hph* gene in the nucleus of tobacco cells to recover glyphosate-tolerant tobacco plants. With nuclear expression, however, glyphosate resistance levels were low in the transgenic plants (perhaps due to the cytosolic localization of HPH).

In 1995, Peñaloza-Vazquez and colleagues (Peñaloza-Vazquez I) described the isolation of a glyphosate-degrading bacterial strain, *Pseudomonas pseudomallei* 22, from glyphosate-treated soil. They further described the cloning and characterization of two genes, *glpA* and *glpB*, which were involved in the degradation of glyphosate. The *glpA* deduced amino acid sequence revealed a significant level of identity to the *E. coli hph* gene, suggesting that *glpA* encoded a phosphotransferase enzyme. This was confirmed when they demonstrated that the *glpA* enzyme could utilize both glyphosate and hygromycin B as a substrate for phosphorylation (like the HPH phosphotransferase). The

glpB DNA and deduced amino acid sequence had no significant homology with any other DNA or protein sequences.

Gene expression studies in *E. coli* revealed that cells harboring *glpA* were able to grow in the presence of 100 µg/ml hygromycin B whereas the host strain was inhibited by a concentration of 50 µg/ml, thus confirming its phosphotransferase activity (Peñaloza-Vazquez I). *E. coli* cells harboring *glpB* alone were able to utilize glyphosate as the sole phosphorous source, suggesting that *glpB* encodes an enzyme with glyphosate-degrading activity (Peñaloza-Vazquez I). Addition of aromatic amino acids was still required, however, to support the growth of *glpB*-expressing *E. coli* cells. This suggested that the *glpB* protein was only able to metabolize sufficient amounts of glyphosate to support the phosphorous requirements of the cell but not relieve the inhibition of aromatic amino acid biosynthesis conferred by the residual amount of intracellular glyphosate. But when *glpB* was co-expressed with *glpA* in *E. coli*, glyphosate was very rapidly degraded. Taken together, these results supported the conclusion that the phosphorylated form of glyphosate was far more readily utilized as a substrate for degradation by the *glpB* protein than the non-phosphorylated form. Although the enzymatic activity of the *glpB* enzyme remains yet uncertain, Peñaloza-Vazquez *et al.* speculated that it probably converts glyphosate by cleavage of the N-C bond to a breakdown intermediate, aminomethylphosphonic acid.

Attempts to create glyphosate-resistant plants through the over-expression of wild-type and mutant forms of EPSPS in transgenic plants have been met with varied degrees of success. Much attention has focused on the generation and characterization of novel EPSPS mutants created at the site of glyphosate binding to identify those forms that were both highly glyphosate-tolerant and still bound the PEP substrate comparably to the wild-type EPSPS (Padgette *et al.*, *J. Biol. Chem.* **266**, 22364 (1991); T. Ruff *et al.*,

Plant Physiol. 96, 94 (1991)). Other studies have identified the critical importance of directing the glyphosate-tolerant EPSPS proteins to the plastid (della-Cioppa *et al.*, *Bio/Technology* 5, 579 (1987) ("della-Cioppa II")).

In 1992, Barry and co-workers (Barry *et al.* in, "Biosynthesis And Molecular Regulation Of Amino Acids In Plants" (B.K. Singh *et al.* [ed.], Am. Soc. Plant Physiologists, Rockville, MD (1992))) described the isolation and characterization of EPSPS from *Agrobacterium* sp. strain CP4. The CP4 EPSPS exhibited very favorable biochemical parameters, namely high glyphosate tolerance and tight binding for PEP, which strongly suggested that expression of the CP4 EPSPS gene in transgenic plants might lead to high levels of glyphosate tolerance. This prediction has been borne out as the CP4 EPSPS gene has been introduced into soybean nucleus to create highly glyphosate-tolerant soybeans (Padgette *et al.*, *Crop Science* **35**, 1451 (1995)), which are now commercially available to farmers as ROUNDUP® READY® soybeans. Zhou and colleagues (Zhou *et al.*, *Plant Cell Rep.* **15**, 159 (1995)) have demonstrated that bombardment with the CP4 EPSPS gene, under control of the duplicated CaMV 35S promoter (Kay *et al.*, *Science* **236**, 1299 (1987)), into non-photosynthetic embryogenic callus resulted in nuclear expression of the bacterial enzyme and permitted the recovery of glyphosate-resistant, transgenic wheat plants.

There has been much work focusing on techniques for transforming the plastid. Cannon *et al.* (EP 0 251 654) generally discusses the transformation of plastid genomes with heterologous DNA employing a selectable marker gene. McBride *et al.* (*Proc. Natl. Acad. Sci. USA* **91**, 7301 (1994)) demonstrated that exceptionally high levels of transgene expression are achievable in plastid transformants. Biolistic transformation (for review, see Sanford *et al.*, *Methods Enzymol.* **217**, 483 (1992)) of the chloroplast genome of the freshwater unicellular green alga, *Chlamydomonas reinhardtii*, was the first report

(Boynton *et al.*, *Science* **240**, 1534 (1988)) of genetic modification of this organellar DNA in any organism. Later, Blowers *et al.* (*Plant Cell* **1**, 123 (1989)) demonstrated that foreign genes could be stably maintained on the chloroplast chromosomes of *Chlamydomonas*. The first transient transformation of chloroplasts in intact higher plant cells was demonstrated in 1990 (Daniell *et al.*, *Proc. Natl. Acad. Sci. USA* **87**, 88 (1990); Ye *et al.*, *Plant Mol. Biol.* **15**, 809 (1990)). Daniell has reviewed higher plant chloroplast transformation (Daniell, *Methods Enzymol.* **117**, 536 (1993)). In 1993, Svab and Maliga (*Proc. Natl. Acad. Sci. USA* **90**, 913 (1993)) extended plastid transformation technology by reporting the stable maintenance of a transgene on *Nicotiana tabacum* (tobacco) chloroplast chromosomes. Very recently, Daniell *et al.* (*Nature Biotech.* **16**, 345 (1998)) employed the *aadA* gene and spectinomycin selection to deliver and over-express EPSPS in tobacco chloroplasts and have shown that this can confer glyphosate tolerance.

To recover photosynthetic tobacco cell plastid transformants, regenerable leaf tissue is bombarded with gold or tungsten microparticles carrying genetic constructs for the expression of selectable marker genes like *aadA* (Svab and Maliga, *supra*) and *nptII* (Carrer *et al.*, *Mol. Gen. Genet.* **241**, 49 (1993)), which confer resistance to the antibiotics, spectinomycin and kanamycin, respectively. After bombardment, tissues are placed onto regeneration medium containing the selective agent to recover antibiotic-resistant plants that express the foreign genes in their chloroplasts. Molecular analysis has revealed that integration of the transgenes occurs by two homologous recombination events (Svab and Maliga, *supra*), which lead to direct replacement of the targeted area within the chloroplast genome by the introduced DNA.

Transgenes that express an easily assayable reporter enzyme like ß-D-glucuronidase (GUS) have helped to elucidate molecular mechanisms that govern gene expression in the chloroplast (Staub and Maliga, *Plant J.* 7, 845 (1995)). In addition,

expression of agronomically-important genes like *Bt* toxin genes from *Bacillus thuringiensis* (McBride *et al.*, *Bio/technology* 13, 362 (1995)) has fueled interest in manipulating the plastid genome for the purpose of crop improvement.

Daniell *et al.* (U.S. Patent No. 5,693,507) and Daniell *et al.* (*Nature Biotech.* **16**, 345 (1998)) disclose methods for transforming plant chloroplasts with exogenous genes, in particular with modified EPSPS genes.

U.S. Patent 5,451,513 to Maliga and Maliga teaches transformation of photosynthetic plant plastids with selectable marker genes that when expressed at sufficient levels renders the plant cell resistant to non-lethal antibiotics such as streptomycin and spectinomycin. The '513 patent stresses the importance of using a non-lethal selection system, whereby cells that contain a small number of transformed plastids are able to survive long enough to enable the transformed plastids to replicate to a sufficient number for the cell to achieve homoplasmy and express the transformed phenotype.

Over the past several years, a variety of different foreign genes have been introduced and expressed in the chloroplasts of only a single higher plant species, tobacco (for review, see Maliga, *Trends in Biotech.* **11**, 101 (1993)). Reports of successful chloroplast transformation have been limited to tobacco in large part due to the high regeneration capability of its leaf tissue. An additional advantage to utilizing leaf tissue includes the presence of a large number of transcriptionally-active chloroplasts per cell.

In most plants, including maize, wheat, rice, cotton, turfgrass and oat, however, plant regeneration is not feasible using leaf tissue. In these species and many others, plant regeneration is most easily accomplished through the route of somatic embryogenesis, which involves non-photosynthetic plant tissue, the most common source of material for genetic transformation and subsequent regeneration. Typically, rates of

plant regeneration in these tissue culture systems do not approach that observed for tobacco leaf tissue. In such non-photosynthetic plant tissues (*e.g.*, embryogenic callus and embryogenic cell suspensions), undifferentiated plastids, or proplastids, are present instead of the fully differentiated and functional chloroplasts that develop in green leaf tissue. Since most plant regeneration regimes must be initiated from non-photosynthetic callus or suspension cells, the range of plants whose plastid genomes can be genetically engineered by existing techniques is greatly limited.

A major factor that has severely limited the genetic manipulation of the chloroplast genomes of many plant species has been the lack of a reliable selective agent (and associated selectable marker gene) for the recovery of plastid transformants. Although the spectinomycin resistance phenotype conferred by expression of the *aadA* gene in the plastid has been useful for the recovery of tobacco plastid transformants, it has not been broadly applicable. For example, in attempts to generate plastid transformants of *Petunia hybrida* (petunia), we and others (Hanson, personal communication) have noted that exposure of petunia explants to spectinomycin interferes with the regeneration process of petunia shoots, thereby preventing the recovery of petunia plastid transformants. Also, recovery of *bona fide* spectinomycin-resistant tobacco plastid transformants has been complicated by the concomitant regeneration of spontaneous tobacco mutants that gain resistance to spectinomycin (Svab and Maliga, *supra*). Finally, it has been very uncertain whether spectinomycin, an antibiotic that acts in plants by inhibiting photosynthesis, would be efficacious in the recovery of plastid transformants after DNA introduction into non-photosynthetic plant tissues (*e.g.*, callus and suspension cells).

SUMMARY OF THE INVENTION

To circumvent problems inherent in the prior art and to expand the range of plant species susceptible to plastid transformation, the present inventors sought to identify a selective agent that would permit a more broad application of plastid transformation technology. We discovered that the non-selective herbicide, glyphosate (*N*-phosphonomethyl-glycine), the active ingredient in ROUNDUP® herbicide (Malik *et al.*, *BioFactors* **2**, 17 (1989)), works remarkably well as a chloroplast selective agent and results in much more efficient selection than spectinomycin selection. Despite the observations that glyphosate is a nonselective herbicide, acts primarily in the plastid, and has been successfully used as a selective agent involving non-photosynthetic tissues for the recovery of nuclear expressed glyphosate-resistant transformants, one could not have been reasonably certain that glyphosate would also have utility in higher plant plastid transformations for selection of transformants or for production of plants having commercially acceptable levels of resistance to glyphosate.

The present invention is based upon our realization that the expression of a gene (or genes) in the plastid to confer herbicide (particularly glyphosate) tolerance in transgenic plants would have significant commercial potential. The exceptionally high levels of transgene expression that are achievable in plastid transformants (McBride *et al.*, *supra*) would be favorable for conferring commercially-acceptable levels of herbicide, particularly glyphosate, tolerance. Moreover, since plastid-borne genes are not normally transmitted through the pollen, but only through the egg, the presence of glyphosate resistance transgene(s) on the chloroplast chromosome only would be a very attractive feature when addressing environmental concerns surrounding the issue of transgene dissemination into related "weedy" species. Furthermore, we realized that since many herbicides act within the plastid, expressing herbicide-inactivating genes in

the plastid would circumvent the need for the transport of nuclear-encoded, cytosol-synthesized enzymes into the plastid. To date, however, a means for achieving the foregoing has remained elusive.

Consistent with the foregoing, the present invention provides methods of transforming the plastid (particularly proplastid) genome of a plant with a nucleic acid comprising one or a plurality of selectable marker genes that confer herbicide resistance to the transform plant cells. More particularly, such selectable marker genes express an enzyme that inactivates an herbicide. Gene expression in plastids/proplastids transformed according to the invention occurs at levels that enable a plant having the transformed plastids/proplastids to survive contact with at least the minimal amount of herbicide that would kill an otherwise similar wild-type plant.

In a preferred embodiment, the method comprises plastid transformation with nucleic acids comprising one or a plurality of genes that express enzymes that inactivate glyphosate, phosphinothricin ("PPT") (which is 2-amino-4-(methyl(hydroxyphosphoryl))butanoic acid, the active ingredient in RELY® and FINALE®), or glufosinate (the ammonium salt of phosphinothricin and the active ingredient in BASTA®). (Because their molecular mechanisms of action are the same for the purposes of this invention, the terms "glufosinate" and "PPT" are used interchangeably herein.) In a preferred embodiment, the methods comprise transformation with nucleic acids comprising genes that express proteins that inactivate glyphosate. The *hph* and *glpA* genes are most preferred. While we have found that the *glpB* gene does not itself confer glyphosate resistance, when co-transfected with either or both of the *hph* and *glpA* genes, the *glpB* gene enhances the degree of glyphosate resistance of the transformed plant cell.

In another preferred embodiment, the methods comprise transformation with nucleic acids comprising genes that express proteins that inactivate the herbicide glufosinate. Particularly preferred are the *bar* and *pat* genes.

As implied before, the nucleic acids that are transfected according to the methods of the present invention can comprise a plurality of genes for expression. The genes can encode the same or different enzymes. Consequently, in a preferred embodiment of this aspect of the invention, the methods utilize nucleic acids comprising a plurality of different genes. In one preferred embodiment, the nucleic acid comprises a first gene, which encodes an enzyme that inactivates a first herbicide, and a second gene. The second gene can be a reporter gene or one that produces another desirable phenotypic characteristic, including, but not limited to, resistance to a second herbicide, resistance to an insect or other pathogenic infection, robustness to adverse environmental conditions, and aesthetically pleasing physical characteristics. In another embodiment, the nucleic acid further comprises a third gene, different from the first two, that encodes another desirable phenotypic characteristic, as described above.

Surprisingly, we have found that despite there being many fewer genome copies in proplastids as compared to differentiated plastids (particularly chloroplasts), the methods of the present invention are capable of transforming these organelles. Moreover, the present invention presents for the first time methods that are capable of transforming non-photosynthetic cells, where the number of proplastid genome copies is often an order of magnitude smaller than photosynthetic cells rich in chloroplasts. For many plant species, transformation via proplastids is the only practical route to successful plastid transformation.

The methods of the present invention extend, to a surprising extent, the range of plant species whose plastid genomes may be transformed. The methods of the invention

can be used to confer commercially-acceptable levels of resistance to the herbicide glyphosate when whole plants are regenerated.

Expression cassettes, for the expression of selectable marker genes, reporter genes, or other genes and nucleic acid constructs of interest in the plastid are also described.

The present invention further comprises multicellular plant tissues (particularly whole plants and calli) whose plastids and/or proplastids have been transformed in accordance with the methods of the invention.

In another aspect, the invention also comprises methods and compounds for the transformation of plastids of non-photosynthetic cells with the *aadA* gene, whose expression product confers resistance to the antibiotic spectinomycin. Further provided by this aspect of the invention are multicellular plant tissues having proplastids transformed with the *aadA* gene. That transformation of proplastids with the *aadA* gene confers resistance to a non-photosynthetic cell (in which the proplastids are found) is a surprising result because spectinomycin is believed to interfere with the photosynthetic process, which, of course, is not active in non-photosynthetic cells.

The foregoing merely summarizes certain aspects of the invention and is not intended, nor should it be construed, as limiting the invention, which is described more fully below. All patents and publications recited herein are hereby incorporated by reference in their entirety.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1. *DNA sequence of the petunia chloroplast 16S rDNA promoter region, transcription initiation site, and petunia rbcL leader sequence and ribosome binding site used in the plastid expression cassettes.* The P_{rrn} fragment includes sequences from the

petunia chloroplast 16S rDNA promoter of the ribosomal RNA operon and the 16S rRNA transcription initiation site. Leader sequences and a ribosome binding site (RBS) based upon the petunia *rbcL* gene are also present. The canonical -35 and -10 elements of the 16S rDNA promoter are underlined. The site of transcription initiation is marked by the asterisks. The RBS element is highlighted in bold while the translational initiation codon is both highlighted in bold and underlined.

Fig. 2. *Diagrammatic representation of the plastid expression cassettes for the expression of foreign genes in non-photosynthetic plastids.* The *reporter-aadA* (pSAN347), *glpB-aadA* (pSCO1) and *hph-aadA* (pSCO2) dicistronic operons, and the *glpB-hph-aadA* (pSCO3) polycistronic operon are under control of the 16S rDNA promoter (P_{rrn}), and the 3′ region from the tobacco plastid psbA gene (T_{psbA}). In pSCO35, the *hph* gene is under the control of the 16S rDNA promoter and the 3′ region from the petunia platid rbcL gene (TrbcL). Although the genes are co-transcribed from the 16S rDNA promoter, each protein-coding region has its own RBS element for efficient translation initiation of separate proteins (as indicated by the arrows).

Fig. 3. *Gene insertion into the tobacco plastid genome.* The plastid targeting fragments from the petunia chloroplast genome found in plasmids pSAN308 (A) and pSAN307 (B) for use in the construction of plasmids pSAN347 (A) and pSCO1-pSCO3 (B), respectively, are shown. After integration of the foreign genes into the tobacco plastid genome by homologous recombination, the transgenic chromosomes will have the physical structures shown for pSAN347 (C), pSCO2 (D) and pSCO3 (E). The striped boxes represent the introduced foreign genes. The arrowheads in (A) - (E) show the direction of transcription. Abbreviations are as follows: B, *Bam* HI; H, *Hinc* II; P, *Pst* I; and S, *Sac* I. Note that the *Hinc* II site shown at the end of ORF70B (other *Hinc* II sites in this region are not shown) is the insertion site for the expression cassettes.

Fig. 4. *Reporter activity in spectinomycin-resistant, pSAN347 tobacco NT1 plastid transformants.* (A) Spectinomycin-resistant NT1 transformants recovered after bombardment with pSAN347 plasmid DNA were incubated in buffer to detect reporter gene activity. Observable indications of reporter gene expression were detected within five minutes after addition of substrate. (B). The pSAN347 transformants manifesting expression of the reporter in (A) were assayed for reporter gene activity. Untransformed NT1 cells (control) and nuclear-transformed, kanamycin-resistant NT1 cell lines (pBI426) expressing the reporter gene under control of the enhanced version of the CaMV 35S promoter were also assayed. The values shown represent the mean of twenty transformants.

Fig. 5. *DNA gel blot analysis of spectinomycin-resistant, pSAN347 tobacco NT1 plastid transformants.* Total cellular DNA was isolated, digested with *Bam* HI, transferred to nylon, and probed with a radiolabeled reporter gene fragment. Note that the reporter gene probe hybridizes to a high-copy 6.3 kb fragment in the transgenic lines (lanes 3-9) and no hybridization is detected in the DNA sample from untransformed cells (lane 2). The signal in lane 1 represents hybridization to the reporter gene-containing restriction fragment that was used for radiolabeling.

Fig. 6. *Correct integration of the pSAN347 plastid expression cassette into the plastid chromosomes in spectinomycin-resistant NT1 plastid transformants.* Total cellular DNA was isolated, digested with *Bam* HI, transferred to nylon, and probed with the radiolabeled 3.3 kb *Bam* HI plastid DNA fragment from pSAN307 that comprises the plastid targeting fragment in pSCO2. Note that the pSAN307 probe hybridizes to a high-copy 3.3 kb fragment in the wild-type chromosomes of untransformed cells (lane 3) and a larger, high-copy 6.3 kb fragment in the transgenic chromosomes of the spectinomycin-resistant NT1 cell lines (lanes 4-7). The signal in lane 1 represents hybridization to the

petunia chloroplast DNA-containing restriction fragment that was used for radiolabeling. Lane 2 is empty.

Fig. 7. *The pSCO2 and pSCO3 glyphosate-resistant tobacco transformants contain high levels of HPH phosphotransferase activity.* Cell-free extracts from glyphosate-resistant cells maintained on glyphosate-containing medium were prepared and assayed for the presence of HPH phosphotransferase activity using glyphosate as the substrate. Extracts were prepared from both glyphosate-resistant tobacco NT1 (NT1) and regenerable (NT-R) calli. Extracts prepared from untransformed cells (controls) grown on medium lacking glyphosate were also assayed.

Fig. 8. *DNA gel blot analysis of glyphosate-resistant, pSCO2 NT1 plastid transformants.* Total cellular DNA was isolated, digested with *Bam* HI, transferred to nylon, and probed with a radiolabeled *hph* fragment. Note that the *hph* probe hybridizes to a high-copy 5.5 kb fragment in the transgenic lines (lanes 3-9) and no hybridization is detected in the DNA sample from untransformed cells (lane 2). The signal in lane 1 represents hybridization to the *hph*-containing restriction fragment that was used for radiolabeling.

Fig. 9. *Correct integration of the pSCO2 plastid expression cassette into the plastid chromosome in glyphosate-resistant NT1 plastid transformants.* Total cellular DNA was isolated, digested with *Bam* HI, transferred to nylon, and probed with the radiolabeled 3.3 kb *Bam* HI petunia chloroplast DNA fragment from pSAN307 that comprises the plastid targeting fragment in pSCO2. Note that the pSAN307 probe hybridizes to a high-copy 3.3 kb fragment in the wild-type chromosomes of untransformed cells (lane 3) and a larger, high-copy 5.5 kb fragment in the transgenic chromosomes of the glyphosate-resistant NT1 cell lines (lanes 4-10). The signal in lane

1 represents hybridization to the petunia chloroplast DNA-containing restriction fragment that was used for radiolabeling. Lane 2 is empty.

Fig. 10. *The P_{rrn} fragment used to direct transcription of the plastid selectable marker and reporter genes does not contain a putative nep promoter.* The DNA sequences of the tobacco (Nt), mustard (Sa), soybean (Gm), spinach (So) and maize (Zm)16S rDNA promoter regions, and the petunia-derived sequence used in the plastid expression vectors (P_{rrn}) are shown. Dashes indicate spaces that were introduced to maximize sequence alignment. The canonical -35 and -10 elements of the *pep* promoter for each plant species are underlined. The sites of transcription initiation from the tobacco *pep* promoter are shown below the asterisks. The putative *nep* promoter identified for the tobacco 16S rDNA gene, and the sequences homologous to this promoter in the other plant species are highlighted in bold. The site of transcription initiation that has been identified in transgenic tobacco plants that lack the *rpoB* subunit of the chloroplast-encoded RNA polymerase is marked by the single, solid dot. The ribosome-binding site and the translation initiation codon in the P_{rrn} fragment are italicized and outlined, respectively. The consensus sequence for the putative *nep* promoter that has been identified by Maliga and colleagues is shown at the bottom. Transcription initiation at the consensus *nep* promoter occurs at one (or more) of the last three A residues marked with the solid dots.

Fig. 11. *Gene insertion into monocot plant plastid genomes.* (A) The plastid targeting fragment from the bentgrass chloroplast genome in plasmid pSCO5 for use in the construction of plasmids pSCO6 - pSCO11 (B) is shown. The arrowheads in (A) and (B) show the direction of transcription. Abbreviations are as follows: S, *Sac* I; and X, *Xba* I. The *Sac* I sites at each end of the plastid DNA fragment in (A) were added during the cloning process and are not necessarily found at these sites in the natural bentgrass

plastid chromosome. Note that the unique *Xba* I site shown downstream from ORF72 is the insertion site for the expression cassettes. The individual components of the expression cassettes shown in (B) are as described for Fig. 2.

Fig. 12. *The glyphosate-resistant pSCO2 and pSCO3 tobacco plants contain high levels of HPH phosphotransferase activity.* Leaf cell-free extracts were prepared from glyphosate-resistant pSCO2 and pSCO3 plants maintained *in vitro* on glyphosate-containing medium, and assayed for the presence of HPH phosphotransferase activity using glyphosate as the substrate. A leaf extract prepared from a untransformed plant (control) grown on medium lacking glyphosate was also assayed.

Fig. 13. *Correct integration of the pSCO2 and pSCO3 plastid expression cassettes into the plastid chromosome in glyphosate-resistant tobacco plants.* Total cellular DNA was isolated from leaves, digested with *Bam* HI, transferred to nylon, and probed with the radiolabeled 3.3 kb *Bam* HI petunia chloroplast DNA fragment from pSAN307 which comprises the plastid targeting fragment in pSCO2 and pSCO3. Note that the pSAN307 probe hybridizes to a high-copy, wild-type 3.3 kb fragment in DNA from an untransformed plant (lane 2). Intense 5.5 kb and 6.4 kb signals can be observed in pSCO2 (lanes 3-5) and pSCO3 (lanes 6-9) transformants, respectively. The signal in lane 1 represents hybridization to the petunia chloroplast DNA-containing restriction fragment which was used for radiolabeling.

Fig. 14. *The pSCO2 and pSCO3 glyphosate-resistant tobacco plants survive after spray application of ROUNDUP® herbicide.* Transplastomic pSCO2 and pSCO3 tobacco plants were acclimated in the greenhouse for several weeks. These plants, along with untransformed control plants which had been maintained similarly, were sprayed with a commercial formulation of ROUNDUP® at rates up to 1.8 kg/ha (equivalent to 72 oz/acre). Figs. 14A and 14B display the results in plants of different size. The

untransformed plants on the right died after treatment with 0.3 kg/ha ROUNDUP®. The pSCO2 (*hph*) and pSCO3 (*hph/glpB*) plants survived application rates of 0.8 kg/ha and 1.2 kg/ha, respectively.

Fig. 15. *The pSCO2 glyphosate-resistant maize BMS cells contain high levels of HPH phosphotransferase activity.* Cell-free extracts were prepared from glyphosate-resistant maize BMS cells maintained on glyphosate-containing medium and assayed for the presence of HPH phosphotransferase activity using glyphosate as the substrate. An extract prepared from untransformed BMS cells (control) grown on medium lacking glyphosate was also assayed.

Fig. 16. *Correct integration of the pSCO6 plastid expression cassette into the plastid genome of glyphosate-resistant maize BMS plastid transformants.* Total cellular DNA was isolated, digested with *Bam* HI, transferred to nylon, and probed with the radiolabeled 1.0 kb *Sac* II bentgrass chloroplast DNA fragment from pSCO5 which comprises the plastid targeting fragment in pSCO6. Note that the pSCO6 probe hybridizes to a high-copy, wild-type 3.2 kb fragment in untransformed cells (lane 2). In contrast to the wildtype, an intense 5.4 kb signal can be observed in all seven pSCO6 (lanes 3-9) transformants. The signal in lane 1 represents hybridization to the bentgrass chloroplast DNA-containing restriction fragment which was used for radiolabeling.

Fig. 17. *Glyphosate-resistant bentgrass calli transformed with pSCO6 and pSCO9 contain an hph-specific PCR product.* Total cellular DNA was prepared from three glyphosate-resistant creeping bentgrass calli and two untransformed calli for PCR amplification. The PCR products were fractionated by agarose gel electrophoresis and visualized by UV illumination after staining with ethidium bromide. The lanes were as follows: lane 1, 1 Kb DNA Ladder; lane 2, no DNA control; lanes 3 and 6, pSCO6-

transformed calli; lane 5, pSCO9-transformed calli; and lanes 4 and 7, untransformed calli. The expected *hph*-specific fragment is shown by the arrow on the left.

Fig. 18. *The pSCO6 bentgrass transformant survives after spray application of ROUNDUP® herbicide.* A pSCO6-transformed bentgrass plant was maintained in the greenhouse for several weeks. After acclimation, this plant, along with untransformed control plant which had been maintained similarly, were sprayed with a commercial formulation of ROUNDUP® at a rate of 0.6 kg/ha (equivalent to 24 oz/acre glyphosate). In this photo, taken 29 days after herbicide application, the dead, untransformed control plant is on the left while the thriving pSCO6 transformant is on the right.

Fig. 19. *Glyphosate-resistant rice calli transformed with pSCO8 and pSCO9 contain an hph-specific PCR amplification product.* Total genomic DNA was prepared from a number of glyphosate-resistant rice calli and an untransformed callus sample. PCR amplification results are shown for five samples: -ve control is untransformed callus, +ve control is plasmid DNA (pSCO8), (3) 1 is a callus line transformed with pSCO8, 2 is another callus line transformed with pSCO8, and 3 is a callus line transformed with pSCO9. Each sample has 3 lanes for aliquots taken out after 10, 20, and 30 cycles of PCR with *hph* primers. PCR amplification detected the presence of an *hph*-specific fragment in each of the glyphosate-resistant calli. No PCR product was observed in the reaction which contained genomic DNA from the untransformed control callus.

Fig. 20. *The glyphosate-resistant pSCO2 avocado cells and papaya plants contain high levels of HPH phosphotransferase activity.* Cell-free extracts were prepared from pSCO2-transformed avocado cells maintained on glyphosate-containing medium and assayed for the presence of HPH phosphotransferase activity using glyphosate as the substrate. An extract prepared from untransformed avocado cells (control) grown on

medium lacking glyphosate was also assayed. Leaf cell-free extracts prepared from pSCO2-transformed papaya plants maintained *in vitro* on glyphosate-containing medium were assayed for the presence of HPH phosphotransferase activity using glyphosate as the substrate. A leaf extract prepared from an untransformed plant (control) grown on medium lacking glyphosate was also assayed.

Fig. 21. *Correct integration of the pSCO2 plastid expression cassette into the plastid genomes in glyphosate-resistant avocado callus and papaya plants.* Total cellular DNA was isolated, digested with *Bam* HI, transferred to nylon, and probed with the radiolabeled 3.3 kb *Bam* HI petunia chloroplast DNA fragment from pSAN307 which comprises the plastid targeting fragment in pSCO2. Note that the pSAN307 probe hybridizes to a high-copy, wild-type ~3.3 kb fragment in DNA from untransformed avocado (lane 2) and papaya (lane 6). Instead of the wild-type fragment, a larger, high-copy ~5.5 kb fragment is detected in DNA samples from transformed avocado cells (lanes 3-5) and papaya plants (lanes 7-9). The signal in lane 1 represents hybridization to the petunia chloroplast DNA-containing restriction fragment which was used for radiolabeling.

Fig. 22. *glpA gene insertion into the tobacco plastid genome.* The *defective glpA* plasmid, pSCO24, contains a mutated **glpA** gene (note the destroyed *Nco* I site represented by the crossed-out *N*) under the control of the plastid *rrn* promoter (hatched box). Plasmid pSCO18, the *corrective* copy, contains a wild-type *glpA* gene but lacks both a plastid promoter and an RBS element. After homologous recombination between shared sequences on pSCO24 and pSCO18 (shown by the dotted lines) to restore functionality to the *glpA* gene, and integration into the plastid genome, the transgenic chromosomes (transplastome) will have the physical structure shown below the heavy arrow. The arrowheads associated with the genes show their direction of transcription.

Abbreviations are as follows: B, *Bam* HI; H, *Hinc* II; and N, *Nco* I. The plastid-like ribosome binding site is abbreviated as RBS. Note that the *Hinc* II site shown at the end of ORF70B (other *Hinc* II sites in this region are not shown) is the insertion site for the expression cassettes.

Fig. 23. *The pSCO24/pSCO18 glyphosate-resistant tobacco transformants contain high levels of glpA phosphotransferase activity.* Cell-free extracts were prepared from transformed NT1 cells maintained on glyphosate-containing medium and assayed for the presence of *glpA* phosphotransferase activity using glyphosate as the substrate. An extract prepared from untransformed cells (control) grown on medium lacking glyphosate was also assayed.

Fig. 24. *Correct integration of the pSCO24/pSCO18 plastid expression cassettes into the plastid chromosome restores glpA function in glyphosate-resistant NT1 transformants.* (A) Total cellular DNA was isolated, digested with both *Bam* HI and *Nco* I, transferred to nylon, and probed with radiolabeled *glpA* DNA. Note that the *glpA* probe hybridizes to 2.1 kb and 1.0 kb fragments only in pSCO24/pSCO18 transformants (lanes 4-7). No hybridization is observed in DNA isolated from untransformed NT1 cells (lane 3). The signal in lane 2 represents hybridization to the *glpA*-containing restriction fragment which was used for radiolabeling. (B) Total cellular DNA was isolated, digested with both *Bam* HI and *Nco* I, transferred to nylon, and probed with the radiolabeled 3.3 kb *Bam* HI petunia chloroplast DNA fragment from pSAN307. Note that the pSAN307 probe hybridizes to a high-copy, wild-type 3.3 kb fragment in untransformed cells (lane 3). Instead of the wild-type fragment, two novel, high-copy 2.6 and 2.1 kb fragments are observed in DNA from the glyphosate-resistant NT1 cell lines (lanes 3-6). The signal in lane 1 represents hybridization to the petunia chloroplast DNA-containing restriction fragment which was used for radiolabeling.

Fig. 25. *bar gene insertion into the tobacco plastid genome.* The defective **hph** plasmid, pSCO56, contains a mutated **hph** gene (note the truncated *hph* coding region) under the control of the plastid *rrn* promoter (hatched box). The plasmid is also largely devoid of flanking chloroplast DNA sequences (compare to pSCO57). Plasmid pSCO57, the *bar*-containing template, contains wild-type *hph* and *bar* genes but lacks a plastid promoter. After homologous recombination between shared *hph* sequences on pSCO56 and pSCO57 (shown by the dotted lines) to restore expression to the *hph* and *bar* genes, and integration into the plastid genome, the transgenic chromosomes (transplastome) will have the physical structure shown below the heavy arrow. The arrowheads associated with the genes show their direction of transcription. Abbreviations are as follows: B, *Bam* HI and H, *Hinc* II. The plastid-like ribosome binding site is abbreviated as RBS. Note that the *Hinc* II site shown at the end of ORF70B (other *Hinc* II sites in this region are not shown) is the insertion site for the expression cassettes.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

First Aspect of the Invention

In one aspect, the invention comprises a method of producing an herbicide-resistant plant cell, the method comprising stably transforming the plastid or proplastid genome of the plant cell with a nucleic acid that comprises a first herbicide-resistance-conferring selectable marker gene, wherein the first herbicide-resistance-conferring selectable marker gene encodes a protein that inactivates the herbicide, and which gene is expressed at levels that result in the plant cell surviving contact with the minimal amount of the herbicide that would kill a untransformed plant cell of the same species.

As demonstrated herein, we have discovered that genes that encode enzymes that inactivate herbicides are extremely effective selectable markers for use in plant plastid transformations. The invention is useful not only as a research and development tool for identifying and selecting plant cells whose plastids and/or proplastids have been successfully transformed, but also for producing plants resistant to an herbicide. The method according to this aspect of the invention is useful for transformation of the plastid genome of any plant species that is amenable to manipulation under tissue culture conditions, including both monocots and dicots. The Examples presented herein demonstrate successful transformation according to the invention of both monocots and dicots as well as of plants having a plastid genome of virtually unknown content.

The method employs selection techniques based on cell survival when cells, which have been subject to transformation protocols described herein, are contacted with an herbicide. We have found that contrary to the teachings of the prior art (as disclosed, for example, in U.S. Patent 5,451,513), plastid transformation need not be conducted under non-lethal selection conditions.

When the herbicide-resistant plant cell produced according to this aspect of the invention is a regenerable cell (which is preferable), multicellular plant tissues resistant to the herbicide can be generated using art recognized methods. Plant tissues produced according to the invention are able to withstand contact with the minimal amount of herbicide that would kill a similar, untransformed plant tissue of the same species. It is a routine matter for the one of ordinary skill in the art to determine the minimal amount of herbicide that would kill a non-transformed plant tissue.

Importantly, however, plant cells and tissues according to the invention can withstand at least twice the concentration (denoted as "2x") of herbicide typically applied in field applications and up to amounts of about 5x. For easy to control plants such as

grasses, about 2 quarts of 41% glyphosate solution per acre are typically used (which is equivalent to 20 gallons of 2.5% ROUNDUP® ULTRA (41% glyphosate) solution per acre). For hard to control plants such as dandelions, about 4-5 quarts of 41% glyphosate solution per acre are typically used.

As used herein, the term "inactivates" means to chemically modify or degrade an herbicide in such a manner as to reduce or eliminate its toxicity to the plant cell. The term, "stably transforming the plastid or proplastid genome of the plant cell with a nucleic acid" means that under desired conditions the transformed plant cell retains the transfected phenotype and does not revert back to the wild-type. In some applications, the cell will be maintained in such a manner so as to allow it to achieve a state of homoplasmy following transfection, and the "desired conditions" are any in which the cell can survive and which exerts a selective pressure favoring growth and multiplication of transformed genomes, plastids, and cells. In other applications, such as commercial applications, the "desired conditions" may be field conditions, with or without periodic application of a herbicide.

Furthermore, as used herein, "stably transforming the plastid or proplastid genome of the plant cell with a nucleic acid" means that subsequent to transformation, the genome contains a non-native nucleic acid; the term is intended to imply nothing as to whether the transformation occurred as a result of recombination of a single nucleic acid into the plastid genome or a plurality of nucleic acids into the genome.

Briefly, and as illustrated in more detail below, plant cells are transfected with a plasmid comprising a region homologous to the plastid genome of the cells and further comprising an expression cassette including the first herbicide-resistance-conferring selectable marker gene, any other genes of interest, and various control elements. Transfection may be accomplished by any convenient technique, *e.g.*, PEG treatment,

electroporation, and biolistic delivery. Biolistic delivery is preferred. The transfected nucleic acid comprising the expression cassette incorporates into the plastid genome through homologous recombination events. The cells are then placed in a medium containing the herbicide (for which the herbicide resistance-conferring gene confers resistance) as well as other necessary nutrients, thereby exerting a selective pressure that favors the growth and replication of transformed genomes, plastids, and cells. Untransformed cells die in this medium (or fail to grow), whereas the transformed cells survive and grow towards a state of homoplasmy (although heteroplasmy may sometimes be desirable and, therefore, sustained).

In practice, the nucleic acid employed in this aspect of the invention can be mono- or polycistronic and preferably comprises not only the herbicide resistance-conferring selectable marker gene, but various control elements as well. Such control elements will preferably include, but are not limited to promoters (*e.g.*, 16S rDNA promoter (*rrn*) ,) and a ribosome binding site (RBS) (*e.g.*, that derived from petunia *rbcL* gene) positioned at an appropriate distance upstream of the translation initiation codon to ensure efficient translation initiation. A chloroplast promoter is preferred. The petunia chloroplast 16S rDNA promoter of the ribosomal RNA operon is particularly preferred.

Additionally, because recombination of the plastid genome occurs by homologous recombination events, the nucleic acid will further comprise flanking sequences (one on each of the 3′ and 5′ ends of the expression cassette) that are homologous to sequences within the plastid genome. The flanking sequences not only facilitate recombination, they also provide the means by which the method selectively targets the plastid genome for recombination. Expression vectors not having sequences homologous to a region within the plastid genome will not recombine with the plastid genome. Preferably, the nucleic acid's 3′-end further comprises a stem-loop structure to

confer stability. A preferred flanking sequence is derived from the tobacco *psbA* 3′-flanking region.

Although originating from dicots, we have found that the petunia *rrn* promoter and tobacco *psbA* 3′-flanking region are both suitable for use in monocot transformations. The same conclusion was also reached for the tobacco T_{psbA} element, which is required for transcript 3′ end maturation and stability, although any other element that provides 3′-end maturation and stability can be used in place of T_{psbA}. For example, elements having stem-loop structures can also be used for transcript 3′ end maturation and stability. Although the use of heterologous (petunia) chloroplast DNA sequences to direct the gene expression cassettes into the tobacco chloroplast chromosome had not been previously described, the high degree of DNA sequence homology between the two plastid genomes in the inverted repeat region suggested that this would not be problematic.

Of the major crops grown in the United States as well as abroad, such as wheat, corn, oats, sorghum and rice, all are classified as monocotyledonous plants. Although the gene content and gene arrangement of plastid genomes are generally very conserved among vascular land plants, there are some significant differences that have been reported. These differences may include genome size, gene content, gene organization, variable spacing between genes, and differing sizes of the single-copy and inverted repeat regions. Therefore, the selection of an insertion site for foreign genes and the associated flanking sequences that surround the insertion site to provide the necessary homology for integration into the plastid chromosome must be carefully considered. The insertion of transgenes into the plastid genome must not disrupt essential chloroplast genes nor seriously interfere with the expression of neighboring genes.

We have found that the inverted repeat region of the plastid genome is a suitable, and preferable, locus for recombinant insertion of the nucleic acids in all aspects of the invention. The entire chloroplast genomes of the monocots, *Oryza sativa* (rice) (Hiratsuka *et al.*, *Mol. Gen. Genet.* **217**, 185 (1989)) and *Zea mays* (maize) (Maier *et al.*, *J. Mol. Biol.* **251**, 614 (1995)) have been sequenced. DNA sequence comparison reveals that these monocot genomes share a very high degree of homology with each other. Inspection of the rice and maize plastid inverted repeat sequences, as a particular example, reveals the shared presence of an intergenic region that contained no detectable protein-coding regions. Fig. 11 depicts this span, nearly 1 kb in length, located between exon 2 of the *rps12* gene and a putative protein-coding region of unknown function designated ORF72. Importantly, the DNA sequence homology in this intergenic region and in the flanking regions is extremely high between maize and rice, suggesting that the flanking regions are suitable for targeting foreign genes into a variety of monocot plastid chromosomes. An additional attractive feature of this region is that, in the unlikely event that DNA integration disrupted an essential chloroplast gene (identified or not), an intact duplicate of this region would still remain on the other copy of the inverted repeat. Under this scenario, plastid transformants would likely be recovered that would be predicted to then contain dissimilar inverted repeat regions.

The rice and maize genomes also share significant homology with the tobacco plastid genome, the first dicot chloroplast genome to be sequenced in its entirety (Shinozaki *et al.*, *EMBO J.* **5**, 2043 (1986). While gene content differences and structural changes exist between the monocots and the dicots, selected portions of the inverted repeat segments of dicot and monocot plastid chromosomes are some of the most highly conserved regions of the plastid genome. Accordingly, and more importantly, as

demonstrated below, the inverted repeat region of the plastid genome is suitable for homologous recombination in both monocots and dicots.

A surprising feature of this aspect of the invention is that it can be employed to transform not only differentiated plastids in photosynthetic cells, but proplastids in non-photosynthetic cells as well. This result is unexpected because non-photosynthetic cells have many fewer plastids than photosynthetic cells, and the proplastid plastome consists of many fewer copies of the plastid genome as compared to differentiated plastids. *E.g.*, Maliga, *Tibtech* 11, 101 (1993). For example, there are 10-15 proplastids per meristem cell (a non-photosynthetic cell), each of which contains about 50 genome copies, to give roughly 500-750 genome copies per cell. By contrast, a leaf cell can contain as many as 100 chloroplasts, each with about 100 copies of the genome, to make about 10,000 genome copies per leaf cell. In some species, this may be as high as 50,000 genome copies per leaf cell. Because of the much larger number of genome copies in photosynthetic cells, prior art attempts at transforming the plastid genome have employed photosynthetic cells. Despite the relative paucity of genome copies in proplastids, we have been able to transform non-photosynthetic cells and generate whole plants therefrom with the methods provided herein.

To our knowledge, the present invention demonstrates for the first time the ability to and utility of transforming non-photosynthetic cells. The importance of this feature can be appreciated when one realizes that nearly all regeneration systems for monocot plants rely upon the initiation and maintenance of regenerable, non-photosynthetic callus or cell suspension cultures.

Out data also suggests that transformation of non-photosynthetic cells is likely more efficient than transformation of photosynthetic cells. In Example 1, *infra*, wherein the *aadA* gene was co-transfected with a reporter gene, 40 out of 40 calli assayed positive

for reporter gene activity. This observation indicates that the background of spontaneous mutation(s) that confer resistance to spectinomycin in NT1 cells is at least 40-fold below plastid transformation rates and are essentially undetectable in this system. This is in marked contrast to the recovery of spectinomycin-resistant plastid transformants after bombardment of tobacco leaves with a similar *aadA*-expressing transgene. In that system, the background of spectinomycin-resistant mutants that are attributed to spontaneous mutations (Svab and Maliga, *supra*) is at least 10-fold higher than observed with NT1 cells. Thus, recovery of *aadA*-expressing, spectinomycin-resistant calli is likely more efficient in non-photosynthetic cell systems than ones that are photosynthetically competent.

Other types of leaf tissue can be transformed according to the invention, however, including callus and leaf tissue. As previously noted, the method can be used to transform monocots as well as dicots, including ornamental plants, turfgrass, soybeans, wheat, cotton, rice, canola, and corn.

Moreover, another surprising advantage of the present method is that no knowledge of the target plastid genome is required, as demonstrated in Example 8, *infra*. In that Example the plastids of avocado and papaya, two relatively obscure and exotic plants that have not been extensively used in transgenic studies, were transformed. Moreover, their plastid genomes are virtually uncharacterized. We demonstrate herein that glyphosate-resistant avocado cell lines and papaya plants with transformed plastid chromosomes can indeed be recovered with relative ease despite the lack of knowledge regarding their plastid genome. These results demonstrate that methods of the present invention can be used for widespread, routine manipulation of the plastid genomes in a diverse array of land plants.

In a preferred embodiment of this aspect of the invention, the nucleic acid comprises the *hph* gene, the sequence of which is disclosed in Gritz and Davies (*Gene* **25**, 179 (1983)) and Santerre and Rao (U.S. Patent No. 4,727,028). The *hph* gene has been shown to phosphorylate both glyphosate and hygromycin. Peñaloza-Vasquez I, *supra*. *Hph* expression in the plant nucleus is insufficient to select for glyphosate resistance, and commercially useful levels are not achieved. We have found that the *hph* gene, when inserted into a nucleic acid expression cassette and transfected into the plastid or proplastid genome expressed in photosynthetic or non-photosynthetic plastids according to this embodiment of the invention, is an excellent selectable marker and allows the recovery of glyphosate-resistant plant cell transformants, from which, if the cell is regenerable, multicellular transformed plant tissues can be generated. Although the invention is not limited by any theory of action, we theorize that glyphosate entering into the plastid is phosphorylated and subsequently inactivated by hygromycin phosphotransferase (HPH, the *hph* expression product), thus permitting the growth of glyphosate-resistant plant cells and leading to whole plants with significant field levels of resistance to glyphosate.

As used herein, "glyphosate" means N-(phosphonomethyl)glycine in free or salt form, preferrably the mono(isopropylamine) salt (*e.g.*, ROUNDUP®) or the trimesium salt (*e.g.*, TOUCHDOWN®).

As demonstrated in Example 2, *infra*, plastid transformation with the *hph* gene results in cells displaying a surprisingly higher level of HPH phosphotransferase activity (up to six times higher) as compared to nuclear-transformed, partially or very weakly glyphosate-resistant cells.

In another preferred embodiment of this aspect of the invention, the nucleic acid comprises the *glpA* gene, the sequence of which is disclosed in Peñaloza-Vazquez I,

supra. Like the *hph* gene, the *glpA* gene has been shown to phosphorylate glyphosate. Peñaloza-Vazquez I, *supra*. The *glpA* gene, when inserted into a nucleic acid expression cassette and transfected into the plastid or proplastid genome expressed in photosynthetic or non-photosynthetic plastids according to this embodiment of the invention, allows the recovery of glyphosate-resistant plant cell transformants, from which, if the cell is regenerable, multicellular transformed plant tissues can be generated.

In still another preferred embodiment of this aspect of the invention, the nucleic acid comprises the *bar* gene, the sequence of which is disclosed in Thompson et al., *EMBO J.* 6, 2519 (1987). The *bar* gene, when inserted into a nucleic acid expression cassette and transfected into the plastid or proplastid genome expressed in photosynthetic or non-photosynthetic plastids according to this embodiment of the invention, allows the recovery of glufosinate-resistant plant cell transformants, from which, if the cell is regenerable, multicellular transformed plant tissues can be generated.

In yet another preferred embodiment of this aspect of the invention, the nucleic acid comprises the *pat* gene, the sequence of which is disclosed in Wohlleben et al., *Gene* 70, 25 (1988). The *pat* gene, when inserted into a nucleic acid expression cassette and transfected into the plastid or proplastid genome expressed in photosynthetic or non-photosynthetic plastids according to this embodiment of the invention, allows the recovery of glufosinate-resistant plant cell transformants, from which, if the cell is regenerable, multicellular transformed plant tissues can be generated.

In another embodiment of this aspect of the invention, the nucleic acid comprises a plurality of genes. In one preferred embodiment, the nucleic acid further comprises a second gene. The first herbicide resistance conferring selectable marker gene and the second gene can encode the same or different enzymes. Generally, however, a single copy of a gene is sufficient to confer a desired phenotype. Consequently, in a preferred

embodiment, the methods utilize nucleic acids comprising a plurality of different genes. When the nucleic acid comprises two genes, the second gene can be a reporter gene or one that produces another desirable phenotype (*e.g.*, one of agronomic interest), including, but not limited to, resistance to a second herbicide, resistance to an insect or other pathogenic infection, robustness to adverse environmental conditions, and aesthetically pleasing physical characteristics, including pleasant aroma and/or appearance. A suitable reporter gene that can be the second gene is the *gusA* gene. Jefferson *et al.*, *EMBO J.* 6, 3901 (1987).

Alternatively, the second gene can be one that enhances the function of the first gene. In a preferred embodiment, the first gene is the *hph* gene or the *glpA* gene and the second gene is the *glpB* gene, the sequence of which is disclosed in Peñaloza-Vazquez I. We have found that cells whose plastids are transformed with and express only the *glpB* gene do not exhibit significant glyphosate resistance. When the *hph* or *glpA* gene is co-transfected with the *glpB* gene, however, transformed cells manifest increased resistance to glyphosate as compared to cells transformed with either the *hph* gene or the *glpA* gene alone. Earlier gene expression studies in *E. coli* suggested that the *glpB* enzyme highly preferred the phosphorylated form of glyphosate over the unmodified form as a substrate for degradation. Thus, when co-transfected, the *hph* or *glpA* gene enzymes appears to phosphorylate glyphosate, thereby providing the preferred substrate for the *glpB* gene enzyme.

In another embodiment, the second gene can be a second (different) herbicide-resistance-conferring selectable marker gene. (Although the second herbicide-resistance-conferring selectable marker gene could be the same gene as the first, there is not seen to be any particular advantage to transformation with such a construct relative to transformation with a construct containing only one copy of the gene.) In one

embodiment, the second gene may confer resistance to the same herbicide, but by a different mechanism (*e.g.*, a modified enzyme for which the herbicide is not a substrate but which otherwise possesses similar activity to the wild-type enzyme and enables normal cell maintenance, growth, and reproduction). For example, the first herbicide resistance-conferring selectable marker gene can be one whose expression product inactivates glyphosate (*e.g.*, the *hph* gene or the *glpA* gene) and the second gene can be one (a) whose expression product is an active enzyme for which glyphosate is not a substrate (*e.g.*, a modified EPSPS enzyme, such as the *aroA* gene (della-Cioppa II, *supra*), the CP4 EPSPS gene (Barry *et al.*, *supra*), Class II EPSPS genes (*e.g.*, U.S. 5,633,435), GA21 mutant gene (used in, *e.g.*, ROUNDUP® resistant corn) (WO 95/06128) or any other glyphosate-resistant EPSPS enzyme), (b) that overexpresses the EPSPS enzyme and thereby enables the plant or cell to survive contact with amounts of herbicide that would otherwise kill the plant or cell, or (c) one encoding the GOX enzyme (which is a glyphosate oxidoreductase; U.S. 5,776,760).

In another embodiment, the first herbicide resistance-conferring gene and the second gene provide resistance to different herbicides. In a preferred embodiment, the first herbicide-resistance-conferring selectable marker gene is a glyphosate resistance-conferring gene and the second is a glufosinate resistance-conferring gene. In a particularly preferred embodiment, the first herbicide resistance-conferring selectable marker gene is the *hph* or *glpA* gene (to confer glyphosate resistance) and the second herbicide resistance-conferring selectable marker gene is the *bar* or *pat* gene (for conferring glufosinate resistance).

In another embodiment, the nucleic acid further comprises a third gene, different from the first two, that encodes a gene for another desirable phenotypic characteristic or a gene that enhances a phenotypic characteristic, as described above. Thus, for example,

in this embodiment the first gene can be the *hph* or *glpA* gene, the second gene can be the *bar* or *pat* gene, a modified EPSPS gene, an overexpressed EPSPS gene, or the *gox* gene, and the third gene can be the *glpB* gene.

In general, those skilled in the art will appreciate that nucleic acids comprising any one of the multiple combinations of the *hph* (with and without the *glpB* gene) and/or *glpA* genes with one or more modified EPSPS genes, overexpressed EPSPS genes, and the *gox* gene can be used according to the invention to transfect plant plastids. Furthermore, any such nucleic acid can further comprise additional genes of interest, including, but not limited to, genes conferring resistance to other herbicides, resistance to an insect or other pathogenic infection, robustness to adverse environmental conditions, and aesthetically pleasing physical characteristics, including pleasant aroma and/or appearance.

In another embodiment of this aspect of the invention, plant cells transformed according to this aspect of the invention may have previously been transformed with one or more other genes or may subsequently be transformed with one or more other genes. Accordingly, rather than simultaneous co-transformation of the first herbicide-resistance conferring gene with one or a plurality of other genes residing in the same expression cassette, the first herbicide-resistance conferring gene (alone or with one or more other genes) can be transfected into the plastid in a separate transformation event, either before or after transformation with one or more other genes.

Alternatively, plastids can be transformed according to this aspect of the invention by simultaneously co-transfecting a first nucleic acid comprising a first herbicide resistance-conferring gene with a second, separate nucleic acid comprising a second gene.

In yet another embodiment, the nucleic acid sequences necessary for herbicide resistance conferring selectable marker gene expression can be present on a plurality of vectors (preferably two), none of which individually is capable of transforming a plastid to express the gene, but all of which, when inserted into the plastid and when present simultaneously in the plastid, undergo recombination resulting in a transformed plastid genome that expresses the herbicide resistance-conferring selectable marker gene and from which can be generated a cell comprising plastids expressing the gene at levels sufficient to confer herbicide resistance to the minimum level of glyphosate that would kill untransformed cells of the same species can be generated.

This embodiment preferably comprises a method of producing an herbicide-resistant plant cell, the method comprising stably transforming the plastid or proplastid genome of the plant cell with a nucleic acid that comprises a first herbicide-resistance-conferring selectable marker gene, wherein the first herbicide-resistance-conferring selectable marker gene encodes a protein that inactivates the herbicide, and which gene is expressed at levels that result in the plant cell surviving contact with the minimum amount of the herbicide that would kill an untransformed plant cell of the same species, and wherein said transforming comprises introduction of a first vector and a second vector into the plastid, wherein

a) the first vector comprises an herbicide resistance-conferring selectable marker gene whose expression product is capable of inactivating an herbicide, but which vector does not comprise one or a plurality of nucleic acid sequences required for recombination into the plastid or proplastid genome, required for expression of the selectable marker gene, or both,

b) the second vector comprises the nucleic acid sequence or sequences not present in the first vector that are required for recombination into the plastid or proplastid genome, required for expression of the selectable marker gene, or both,

and wherein the first vector, the second vector, and the plastid genome together are capable of recombining through a series of recombination events to produce a plastid genome transformed with the herbicide resistance-conferring selectable marker gene.For instance, Example 9 demonstrates co-bombardment of plastids with two different plasmids. The first plasmid comprises the *glpA-aadA*-T_{psbA} cassette flanked at its 3′ end with petunia chloroplast DNA for facilitating DNA integration into the plastid chromosome, but lacking (a) a 5′ plastid-homologous flanking sequence, (b) a plastid promoter, and (c) a plastid-like RBS element for efficient transcription and translation, respectively. This plasmid, when introduced alone into the plastid, does not confer glyphosate resistance since the *glpA* gene lacks these elements. Moreover, double homologous recombination events between the plasmid and the plastid chromosome leading to integration should occur rarely, if at all, since the gene cassette is flanked on only one side with chloroplast DNA sequences.

The second plasmid comprises the P_{rrn}-**glpA**-*aadA*-T_{psbA} expression cassette, which contains the required homologous flanking sequences and control elements, but which has a defective *glpA* gene (denoted "**glpA**").

Simultaneous introduction of the two plasmids into the plastid resulted in a series of recombination events that resulted in a transformed plastid capable of expressing the *glpA* gene at sufficient levels to confer glyphosate resistance.

In another preferred embodiment, the first plasmid comprises an expression cassette that itself comprises a truncated *hph* plasmid (denoted **hph**) under the control

of the plastid *rrn* promoter. The expression cassette is also largely devoid of flanking chloroplast DNA sequences. The second plasmid comprises wild-type *hph* and *bar* genes and flanking homologous regions but lacks a plastid promoter.

Accordingly, using this approach, plastids can be transformed with herbicide resistance-conferring genes according to the invention by simultaneous introduction of two plasmids into a plastid. Preferably the first plastid comprises an expression cassette lacking both one or more control elements and a homologous flanking sequence at either the 3′ end of the expression cassette or on the 5′ end (but not at both ends). The second plasmid comprises what would otherwise be a suitable expression cassette for transforming a plastid to express the herbicide resistance-conferring selectable marker gene except that the marker gene is defective and, therefore, unable to express an active enzyme. Introduction of both plasmids into the cell (preferably simultaneously) in conjunction with the application of selective pressure (*e.g.*, by exposing transfected cells to an herbicide-containing medium) results, after a series of recombination events, in the production of cells containing plastids transformed with an expressible herbicide-resistance conferring selectable marker gene.

In an alternative embodiment, both plasmids may lack sufficient homologous regions to enable each to individual recombine into the plastid genome, but together recombine to yield a plasmid capable of recombining into the plastid genome to yield a transformed plastid.

This approach offers a couple of advantages. First, it enables introduction and expression of transgenes into plastids that otherwise might not have been possible. Second, it enables the ability to introduce larger segments of foreign DNA in the plastid chromosome. This method effectively doubles the size limit of foreign DNA that can be integrated into the plastid genome in a single transformation event.

In a closely related embodiment, the invention provides a method of transforming a plastid genome with two or more plasmids, each comprise one or a plurality of genes targeted (via homologous nucleic acid regions) to *different* loci in the plasmid genome in a single transformation event. Preferably, one of the genes on one of the plasmids is an herbicide-resistance selectable marker gene (preferably *hph* or *glpA*). Preferably, the other plasmid also comprises a selectable marker gene so that selection for both phenotypes can be made.

To the extent that nuclear inheritance may be desirable, in an alternative embodiment glyphosate resistance can be achieved by transforming the plant cell nucleus with a construct that expresses *hph* or *glpA* (alone or co-expressed with *glpB*), or any of the combinations of genes describe herein, fused with a transit peptide at the 5′ end, which transit peptide targets the expression product to the plastid, particularly the chloroplast. In this manner plastid expression is achieved through nuclear transformation. Numerous methods and constructs for transforming plant cell nuclei are known by those skilled in the art and can be employed. Similarly, several suitable transit peptides for targeting plastids are known by those skilled in the art, as are their coding sequences. Based upon these teachings and those disclosed herein, it would be a routine matter for one skilled in the art to prepare suitable nucleic acid constructs and insert them into plant cell nuclei, resulting in expression of glyphosate resistance-conferring enzymes (alone or with other desired proteins) and their localization in plastids. Such nuclear transformation enables a wider range of options for transcriptional regulation. Furthermore, this embodiment provides Mendelian inheritance.

Second Aspect of the Invention

In a second aspect of the present invention, nucleic acid constructs are provided for use in the first aspect of the invention. The structural features of the nucleic acid constructs according to this aspect of the invention are detailed in the description of the nucleic acids presented in the description of the first aspect of the invention, *supra*. Such nucleic acids can be made using art recognized techniques.

Since the structure and function and the biochemistry and molecular biology of plastids are so highly conserved in both dicotyledonous and monocotyledonous plants, the gene expression cassettes for use in the invention have equal utility in both types of higher plants. This is particularly true for the proplastids that are found in the callus and suspension cells derived from dicot and monocot plants alike.

Because in certain embodiments of the first aspect of the invention successful transformation is accomplished by simultaneous introduction of two plasmids into the plastid, another embodiment of this invention comprises a composition of two vectors,

a) the first vector comprises an herbicide resistance-conferring selectable marker gene whose expression product is capable of inactivating an herbicide, but which vector does not comprise one or a plurality of nucleic acid sequences required for recombination into the plastid or proplastid genome, required for expression of the selectable marker gene, or both, and

b) the second vector comprises the nucleic acid sequence or sequences not present in the first vector that are required for recombination into the plastid or proplastid genome, required for expression of the selectable marker gene, or both,

such that when the composition is introduced into the plastid, the first and second vector, together with the plastid genome recombine to yield a transformed plastid genome capable of expressing the herbicide resistance-conferring selectable marker gene at levels sufficient to confer herbicide resistance to amount of the herbicide that would kill an untransformed cell of the same species.

Compositions according to this embodiment are useful in all embodiments of the first aspect of the invention.

Third Aspect of the Invention

In a third aspect, the invention comprises a cell or cells and multicellular plant tissue (preferably whole plants, calli, and leaf tissue) having cells whose plastid and/or proplastid genomes comprise a first herbicide-resistance-conferring selectable marker gene (preferably a glyphosate resistance-conferring gene; more preferably the *hph* or *glpA* gene), wherein the first selectable marker gene encodes a protein that inactivates the herbicide, and which gene is expressed at levels sufficient to enable the plant tissue to survive contact with the minimal amount of the herbicide that would kill an untransformed plant tissue of the same species.

All of the cells of the multicellular plant tissue comprise plastids transformed with a first herbicide resistance-conferring selectable marker gene, which plastids express the gene at sufficient levels to confer the cell with resistance to the herbicide. The cells can be homoplasmic or heteroplasmic. Preferably the cells are homoplasmic.

The multicellular plant tissue according to this aspect of the invention can be made by transforming the plastids of a regenerable cell using the methods of the first aspect of the invention and then subjecting the cell to art recognized conditions that facilitate its reproduction, differentiation, and growth into a multicellular tissue.

Regeneration of intact plants may be accomplished either with continued selective pressure or in the absence of selective pressure if homoplasmy has already been achieved within the transformed cell line.

In general, multicellular plant tissues according to this aspect of the invention broadly encompass all multicellular plant tissues that can be generated from regenerable cells transformed according to the first aspect of the invention. Thus, for example, multicellular plant tissues according to this aspect of the invention will comprise cells transformed with one, two, three, or more genes, at least one of which is an herbicide resistance-conferring selectable marker gene that inactivates an herbicide. The plant tissue can be monocotyledonous or dicotyledonous and the cells of the tissue photosynthetic and/or non-photosynthetic, homoplasmic, or heteroplasmic.

Fourth Aspect of the Invention

In a fourth aspect, the invention comprises a method of transforming non-photosynthetic cells with the *aadA* gene, a selectable marker gene that confers resistance to the antibiotic spectinomycin. The bacterial *aadA* gene encoding aminoglycoside 3′-adenylyltransferase inactivates spectinomycin, and has already been successfully expressed in photosynthetic tobacco cells to recover plastid transformants (Svab and Maliga, *supra*). We have surprisingly found that the *aadA* gene, when expressed in *non-photosynthetic* plastids, permits the recovery of spectinomycin-resistant plant cell transformants. This is unexpected because the reported mechanism of action of the *aadA* gene product in plant cells is inhibition of photosynthesis.

Expression of a second (or third, etc.) gene, such as a reporter gene or a gene of agronomic interest, can also be accomplished by including that gene on the same plasmid as the *aadA* gene, even within the same transcription unit as the *aadA* gene (polycistronic

operon). Alternatively, the second (or other) gene can be present on a separate vector that is co-introduced with the *aadA*-containing vector.

In general, the method according to this aspect of the invention is the same as for the first aspect of the invention except that (a) the first herbicide resistance-conferring gene of the first aspect is replaced in this aspect of the invention with the *aadA* gene and (b) selection is conducted by exposure of cells to spectinomycin rather than an herbicide.

Similarly, this aspect of the invention also comprises multicellular plant tissues comprising a proplastid transformed with the *aadA* gene.

Having described the present invention, reference will now be made to certain examples, which are provided solely for purposes of illustration and are not intended to limit the invention in any manner. It will be apparent to those skilled in the art that changes and modifications may be made to the above-described and below-exemplified embodiments without departing from the spirit and scope of the present invention.

The protocols described in the following Examples are illustrative for making and using each of the aspects of the invention described above. Following these protocols and relying solely on common knowledge available to one of ordinary skill in the art, one can successfully make and use all aspects of the invention using only but routine experimentation. That is, one would be able to transform *any* plastids of *any* cells according to the methods of the invention and to make the full range of nucleic acids and plants according to the invention.

EXAMPLES

Example 1

Recovery of Spectinomycin-Resistant Tobacco NT1 Cell Plastid Transformants

To assess the efficacy of the *aadA* gene as a selectable marker for plastid transformation in non-photosynthetic cells, a plastid gene expression cassette suitable for foreign gene expression in this organelle was constructed. The *aadA* gene was placed under the control of the strong, constitutive 16S rDNA promoter and the expression cassette embedded within a segment of the petunia chloroplast inverted repeat region to provide DNA sequence homology for recombination events with the resident tobacco plastid chromosomes.

As described more fully below, spectinomycin-resistant calli were recovered in large numbers. DNA gel blot analysis confirmed that the introduced *aadA* gene had integrated into the tobacco plastid chromosome at the expected site by homologous recombination. Moreover, no wild-type plastid chromosomes were detected in the spectinomycin-resistant NT1 transformants indicating that homoplasmy had been achieved. Foreign gene expression in the plastid was further demonstrated by the detection of high levels of enzyme activity from the reporter gene that was contained within the same plastid gene expression cassette.

Materials and Methods for Example 1

Plasmid construction. A chimeric *aadA* expression cassette containing a reporter gene was constructed by placing the reporter gene and *aadA* genes under control of the petunia 16S rDNA promoter. A RBS derived from the petunia *rbcL* gene was provided for efficient translation initiation of the reporter gene. The *aadA* gene, supplied with a nearly-identical RBS element, was inserted immediately downstream of the reporter gene

so that both genes would be co-transcribed. A stem-loop structure from the 3′ end of tobacco *psbA* gene was placed downstream of the *aadA* gene for transcript stability and efficient maturation of the dicistronic transcript's 3′ end. Plasmid pSAN308 contains a 5.8 kb *Pst* I/*Sac* I fragment from the inverted repeat region of the petunia chloroplast chromosome spanning from the *rps7* gene to the *trnA* gene. The dicistronic reporter-*aadA* expression cassette was embedded within the petunia plastid DNA fragment at a *Hinc* II site (to create plasmid pSAN347) such that the transgenes were flanked by ~2.4 kb on the side of the *rps7/rps12* genes and by ~3.4 kb on the side of the *trnI/trnA* genes. Zoubenko *et al.* (*Nucl. Acids. Res.* **22**, 3819 (1994)) had previously demonstrated that this site at the end of ORF70B could be utilized as an insertion site for foreign genes in the tobacco chloroplast genome. The reporter-*aadA* genes are transcribed toward the *rps7/rps12* genes.

Plant cell transformation. Tobacco NT1 suspension cells were collected onto filter paper and placed onto solid NT1 medium containing either 0.4 M mannitol or a combination of 0.2 M sorbitol/0.2 M mannitol for at least 6 hours prior to bombardment. For bombardment, M-10 tungsten particles were coated with pSAN347 plasmid DNA, and introduced into NT1 suspension cells using the PDS1000He Biolistic gun at 800 psi. NT1 cells were allowed to recover overnight on the osmoticum-containing medium and then transferred to medium lacking osmoticum the following day. On the second day following bombardment, the filter paper containing the cells was transferred to NT1 medium containing 500 μg/ml spectinomycin. Spectinomycin-resistant NT1 calli selected for further analysis were maintained on either solid or liquid NT1 medium containing 500 μg/ml spectinomycin.

DNA gel blot analysis. Total cellular DNA was prepared, digested with restriction endonuclease *Bam* HI, and transferred to nylon. Hybridization to a random-primed labeled DNA fragment was carried out overnight at 65 °C.

Results and Discussion

Previously, Svab *et al.* (Svab and Maliga, *supra*) had demonstrated that the *aadA* gene, under control of the tobacco 16S rDNA promoter, was able to confer spectinomycin resistance in photosynthetic tobacco plastid transformants. We had previously constructed a similar plastid gene expression cassette that employed the 16S rDNA promoter region from the highly homologous petunia chloroplast genome (Fig. 1). A DNA fragment containing the strong, constitutive petunia 16S rDNA promoter and transcription initiation site of the ribosomal RNA (*rrn*) operon was cloned. A 5′ leader sequence and a ribosome binding site (RBS) positioned at the appropriate distance upstream of the translation initiation codon to ensure efficient translation initiation were derived from the petunia *rbcL* gene. A reporter gene was inserted next to the RBS (Fig. 2). An *aadA* gene, flanked at its 5′ end with an RBS element based upon the tobacco *rbcL* gene and at its 3′ end by DNA sequences from the 3′ end of the tobacco *psbA* gene, was placed immediately adjacent to the reporter gene. In this gene expression cassette, the reporter and *aadA* genes are co-transcribed as a dicistronic mRNA (Fig. 2), but translation should be initiated at their respective ATG initiation codons since each gene possess its own ribosome-binding site. It had previously been reported (Staub and P. Maliga, *Plant J.* 7, 845 (1995)) that transgenes contained on polycistronic mRNAs were efficiently translated as individual proteins in tobacco chloroplasts.

Since plastid transformation is known to be mediated by homologous recombination events, the reporter-*aadA* expression cassette was embedded with a region

of the petunia chloroplast chromosome (Bovenberg *et al.*, *Nucl. Acids Res.* **9**, 503, 1981). This plasmid had originally been designed for plastid transformation of petunia, but the very high degree of DNA sequence homology between the tobacco and petunia chloroplast genomes in this region suggested to us that the extent of homology would be sufficient for efficient homologous recombination. A 5.8 Kb *Pst* I/*Sac* I fragment from the inverted repeat region of the petunia chloroplast chromosome spanning from the *rps7* gene to the *trnA* gene was cloned (Fig. 3A). Previously, Zoubenko *et al.*, *supra*, had demonstrated the existence of a site located at the end of the ORF70B gene (Shinozaki *et al.*, *EMBO J.* **5**, 2043 (1986)) that was suitable for insertion of foreign genes into the tobacco chloroplast chromosome. The reporter-*aadA* expression cassette was inserted into this site with the direction of transcription toward the *rps7/rps12* genes. Zoubenko *et al.*, *supra*, had further demonstrated that little, if any, readthrough of plastid transcripts occurred in this region of the tobacco chloroplast genome. The high degree of homology with respect to gene sequence and gene arrangement in this region of the petunia chloroplast chromosome strongly suggested to us that the same would hold true in petunia as well. The resulting plasmid, pSAN347, was introduced by particle bombardment into tobacco NT1 cells, where the reporter-*aadA* genes would be expected to integrate into the resident tobacco plastid chromosome(s) via homologous recombination events in the flanking plastid DNA.

Tobacco NT1 suspension cells were bombarded with pSAN347 and allowed to recover for two days prior to being transferred to selective medium containing 500 μg/ml spectinomycin. Within three weeks after bombardment, micro-calli were observed to be growing against a lawn of dead and dying cells. After several more weeks, the calli were picked and transferred to fresh medium containing 500 μg/ml spectinomycin where they continued to grow. An average of approximately 20 spectinomycin-resistant calli per

bombarded plate were observed. No calli were recovered on non-bombarded cells, which were subsequently incubated on spectinomycin-containing medium.

To determine if any of the spectinomycin-resistant NT1 calli expressed the reporter gene, small samples of calli were transferred to microfuge tubes filled with substrate-containing buffer. Within minutes after addition, the calli began to manifest reporter gene expression (Fig. 4A). In total, 40 out of 40 calli manifested the presence of active reporter gene enzyme. No reporter gene expression was ever observed in untransformed NT1 cells.

To gain a more quantitative measurement of reporter activity, cell-free extracts from pSAN347-transformed calli were assayed using a second substrate. As can be observed in Fig. 4B, very high levels of reporter gene expression product activity were observed in the pSAN347-transformed calli, confirming the results obtained with histochemical assays. A comparison of enzyme levels to a nuclear NT1 transformant expressing the reporter gene product was also made.

Tobacco NT1 transformants expressing the reporter gene under control of the enhanced version of the CaMV 35S promoter (E35S) (Kay *et al.*, *supra*) were also analyzed. The plasmid used, harboring the E35S-reporter gene, also included the 5′ untranslated leader region from the alfalfa mosaic virus genome, which serves to increase the translational efficiency of the reporter gene-containing transcript. Thus, this reporter transgene can be considered to be optimized for high levels of nuclear gene expression in tobacco cells. Enzymatic assays revealed that the transformants expressed the reporter gene product at levels approximately 3-fold higher than that observed for pBI426 transformants. Taken together with the histochemical data, these results strongly suggested that the *reporter gene-aadA* dicistronic operon was being highly expressed from the 16S rDNA promoter in the proplastids of NT1 cells.

DNA gel blot analysis of the pSAN347 transformants provided evidence that the *reporter-aadA* genes had integrated into the tobacco plastid genome. If integration into the plastid chromosome has occurred, a single, high-copy 6.3 kb *Bam* HI fragment should be present in pSAN347 transformants (Fig. 3C). Total cellular DNA isolated from seven spectinomycin-resistant NT1 transformants was digested with *Bam* HI and probed with the reporter gene. As can be observed in Fig. 5, a single 6.3 kb reporter-hybridizing *Bam* HI fragment (lanes 3-9) measured to be present in 500-1,000 copies per cell was detected. No hybridization to the DNA sample from untransformed NT1 cells was observed (lane 2).

If the reporter-*aadA* expression cassette has inserted into the expected chromosomal location by homologous recombination, the wild-type 3.3 kb *Bam* HI fragment should be replaced by a larger, novel 6.3 kb *Bam* HI fragment when probed with petunia chloroplast DNA from plasmid pSAN307 (Fig. 3C). In DNA from untransformed NT1 cells, the expected 3.3 kb *Bam* HI fragment was detected (Fig. 6, lane 3). However, in the four spectinomycin-resistant lines that were examined (from the seven in Fig. 5), the anticipated 6.3 kb *Bam* HI fragment was detected (lanes 4-7), indicating correct integration at the expected chromosomal location. No wild-type 3.3 kb *Bam* HI fragment was detected in any of the four lines. The lack of wild-type fragments illustrates two important points. First, the reporter-*aadA* cassette has been "copy-corrected" from one copy of the inverted repeat to the other inverted repeat. Second, all the chloroplast chromosomes have been transformed, indicating that homoplasmy has been achieved.

Taken together with the reporter gene product expression data, these results provide convincing evidence that the *aadA* gene can be utilized to recover plastid transformants in non-photosynthetic cells like NT1. As noted previously, *a priori* it was

uncertain whether spectinomycin would be an effective selective agent for the recovery of plastid transformants of non-photosynthetic cells since its reported mechanism of action in plant cells is inhibition of photosynthesis. The results presented herein are consistent with the notion that spectinomycin could also disrupt protein synthesis in mitochondria, another organelle with prokaryotic-like (70S) ribosomes. If so, high levels of gene expression from the plastid-borne *aadA* gene may be sufficient to inactivate enough spectinomycin that enters into the cell and plastid to provide protection to the mitochondria as well, thus permitting growth.

Example 2

Recovery of Glyphosate-Resistant Tobacco NT1 Plastid Transformants

As described in Example 1, recovery of plastid transformants of tobacco NT1 cells, a non-photosynthetic cell line was achieved. To determine if plastid transformants could be recovered using an alternative selectable marker gene, the *hph* gene was investigated for its ability to confer resistance to the herbicide, glyphosate. The *hph* gene was placed under the control of the strong, constitutive 16S rDNA promoter and the expression cassette embedded with a segment of the petunia chloroplast inverted repeat region to provide DNA sequence homology for recombination events with the resident tobacco plastid chromosomes. Glyphosate-resistant calli were recovered in large numbers. High levels of HPH phosphotransferase activity were detected in the glyphosate-resistant NT1 transformants. DNA gel blot analysis confirmed that the introduced *hph* gene had integrated into the tobacco plastid chromosome at the expected site by homologous recombination. Moreover, no wild-type plastid chromosomes were detected in the glyphosate-resistant NT1 transformants indicating that homoplasmy had been achieved. Bombardment of regenerable, photosynthetically-active tobacco callus

has also resulted in the recovery of glyphosate-resistant calli that contain HPH phosphotransferase activity. The novel genetic construct described herein may be used to extend the range of species in which plastid transformation is feasible and may be used to recover glyphosate-resistant plants with commercially-acceptable levels of herbicide resistance.

Materials and Methods for Example 2

Plasmid construction. A chimeric *hph-aadA* expression cassette was constructed by placing the *hph* and *aadA* genes under control of the petunia chloroplast 16S rDNA promoter. An RBS derived from the petunia *rbcL* gene was placed 3′ to the transcription initiation site for efficient translation initiation of the *hph* gene. The *aadA* gene, supplied with a nearly-identical RBS element, was inserted immediately downstream of a reporter gene so that both genes would be co-transcribed. A stem-loop structure from the 3′ end of tobacco *psbA* gene was placed downstream of the *aadA* gene for transcript stability and efficient maturation of the dicistronic transcript's 3′ end. This dicistronic cassette was embedded within petunia plastid DNA sequences at the same *Hinc* II site as described in Example 1. However, rather than the 5.8 kb *Pst I/Sac* I fragment in pSAN308, a 3.3 kb *Bam* HI sub-fragment of this region found in plasmid pSAN307 was employed. This *Bam* HI fragment from the inverted repeat region of the petunia chloroplast chromosome spans from beyond the ORF70B gene to the *trnI* gene such that the *hph-aadA* cassette was flanked by ~0.9 kb on the side of the ORF70B gene and by ~2.4 kb on the side of the *trnV*-16S rDNA-*trnI* genes. The direction of transcription of the *hph-aadA* dicistron is toward the ORF70B/*rps12*/*rps7* genes.

Plant cell transformation. Plant cell transformation was carried out as described in Example 1 except that the bombarded cells on filter paper were transferred to selective

medium containing either 1 mM or 2 mM glyphosate. Both levels were equally efficacious in the recovery of glyphosate-resistant transformants.

Phosphotransferase assays. Cell extracts prepared from calli were tested with glyphosate for phosphorylating activity with [-^{32}P]ATP as described first by Haas and Dowding (*Methods Enzymol.* **43**, 611 (1975)) and later by Peñaloza-Vazquez *et al.* (Peñaloza-Vazquez I).

Gel blot analysis. DNA gel blot analysis was carried out as described in Example 1.

Results and Discussion

Peñaloza-Vazquez I recently demonstrated that hygromycin phosphotransferase, the product of the *hph* gene, efficiently utilized glyphosate as a substrate for phosphorylation. Moreover, *E. coli* cells harboring the *hph* gene were able to grow in glyphosate-containing medium. These observations prompted them to investigate the possibility that the *hph* gene might confer glyphosate resistance in transgenic plants. Although the *hph* gene has been widely used as a selectable marker gene in nuclear plant transformation studies to confer resistance to the antibiotic, hygromycin B, whether resistance to glyphosate could be attained was unknown. Indeed, expression of the *hph* gene in the nucleus conferred low, but detectable levels of glyphosate resistance in transgenic tobacco plants (Peñaloza-Vazquez II). We sought to extend these observations to determine if the *hph* gene, when expressed in the plastid, could be utilized as a selectable marker gene for plastid transformation.

The *hph* gene was placed under control of the strong, constitutive petunia chloroplast 16S rDNA promoter (Fig. 2). An *aadA* gene was situated immediately downstream of the *hph* gene such that a dicistronic transcript would be expected to be

synthesized by the plastid RNA polymerase. A stem-loop region from the tobacco *psbA* gene was provided at the 3′ end of the transcript for mRNA 3′ end maturation and transcript stability. For plastid targeting, a 3.3 kb *Bam* HI sub-fragment from the petunia DNA insert found in pSAN308 was utilized (Fig. 3B). This *Bam* HI fragment from the inverted repeat region of the petunia chloroplast chromosome spans from beyond the ORF70B gene to the *trnI* gene such that the *hph-aadA* cassette was flanked by ~0.9 kb on the side of the ORF70B gene and by ~2.4 kb on the side of the *trnV*-16S rDNA-*trnI* genes. The direction of transcription of the *hph-aadA* dicistron is toward the ORF70B/*rps12*/*rps7* genes. This plasmid was designated pSCO2.

Plasmid pSCO2 was precipitated onto tungsten microparticles and bombarded into tobacco NT1 cells for selection on either 1 mM or 2 mM glyphosate-containing medium. Within 2-3 weeks after transfer to selective medium, microcalli were observed. Within 5-6 weeks after bombardment, these calli were transferred to fresh medium containing 2 mM glyphosate. An average of approximately 100 glyphosate-resistant calli per bombarded plate were observed. No calli were ever observed on plates of non-bombarded cells. After allowing the calli to proliferate, cell suspensions were established from a number of independently-transformed calli and the cell lines challenged with higher concentrations of glyphosate. Cells continued to grow in liquid medium containing 10 mM glyphosate, the highest level tested.

Phosphotransferase assays were carried out to detect the enzymatic activity of the HPH protein. Cell-free extracts were prepared from the glyphosate-resistant transformants and tested for their ability to phosphorylate glyphosate *in vitro*. As can be observed in Fig. 7, high levels of HPH phosphotransferase activity were detected in all three pSCO2 NT1 transformants. For comparison, Peñaloza-Vazquez *et al.* (Peñaloza-Vazquez II) reported a lower level of HPH phosphotransferase activity (2.79 x 10^3

cpm/mg protein) in leaf extracts of a nuclear-transformed, glyphosate-resistant tobacco plant (their strongest HPH expressor). Little phosphotransferase activity was detected in the extract prepared from untransformed NT1 cells.

DNA gel blot analysis was carried out to determine if the *hph-aadA* cassette had inserted into the plastid genome. Total cellular DNA was isolated, digested with *Bam* HI, and probed with radiolabeled *hph* DNA. As can be observed in Fig. 8, the expected 5.5 kb *Bam* HI fragment was observed in all seven glyphosate-resistant cell lines. The copy number of this fragment was measured to be approximately 500-1,000 copies per cell. If the *hph-aadA* cassette has inserted into the expected chromosomal location, the wild-type 3.3 kb *Bam* HI fragment should be replaced by a larger, novel 5.5 kb *Bam* HI fragment when probed with petunia chloroplast DNA from plasmid pSAN307 (Fig. 3D). In DNA from untransformed NT1 cells, the expected 3.3 kb *Bam* HI fragment was detected (Fig. 9). However, in all seven glyphosate-resistant lines, the anticipated 5.5 kb *Bam* HI fragment was detected, indicating correct integration at the expected chromosomal location. No wild-type 3.3 kb *Bam* HI fragment was detected in any of the seven lines. The lack of wild-type fragments illustrates two important points. First, the *hph-aadA* cassette has been "copy-corrected" from one copy of the inverted repeat to the other inverted repeat. Second, all the chloroplast chromosomes have been transformed, indicating that homoplasmy has been achieved in each of the seven lines examined. Taken together, these results indicate that glyphosate is an extremely efficient selective agent in the recovery of plant cell plastid transformants and at promoting the establishment of homoplasmy in these transformants.

Glyphosate selection of plastid transformants expressing *hph* should be equally efficacious in regeneration systems that utilize photosynthetic or non-photosynthetic cells as the recipient tissue for introduction of foreign genes. To support this argument,

bombardment of pSCO2 into a regenerable, photosynthetically-active tobacco cell suspension has resulted in the recovery of glyphosate-resistant green calli. Fig. 7 shows that one pSCO2 transformant (NT-R) contained HPH phosphotransferase activity similar to that observed for the pSCO2 NT1 transformants. Although sufficient plant material for DNA gel blot analysis was not yet available, there is every reason to believe that the *hph-aadA* expression cassette has integrated into the chloroplast chromosome at the expected site, as was found for the pSCO2 NT1 transformants.

Moreover, the *hph* gene, when expressed in the plastid, confers high levels of glyphosate resistance to plastid transformants (transformed cells continue to grow in the presence of 10 mM glyphosate, the highest level tested). Even higher levels of plastid-localized HPH phosphotransferase activity (and glyphosate resistance) should be achievable in the photosynthetically-active chloroplasts found in leaf tissue as a number of factors act together to dramatically boost chloroplast gene expression activity in green tissue. These factors include an increase in the number of chloroplasts per cell, higher numbers of chromosomes due to increases in chloroplast number as well as chromosomes per chloroplast, and an overall up-regulation in transcriptional/translational activity throughout the genome.

Commercially-acceptable levels of glyphosate resistance should be achievable in regenerated plants that express the *hph* gene in their plastids. Even in non-photosynthetic tissues like meristems and roots, *hph* gene expression levels should be comparable to that observed with a nuclear construct driven by the very active enhanced version of the CaMV 35S promoter (see Example 1). The availability of glyphosate-resistant pSCO2 tobacco plants in the near future (regenerating from the green calli) will permit a comprehensive analysis of the glyphosate resistance levels that have been achieved.

Example 3

Recovery of Glyphosate-Resistant Tobacco NT1 Plastid Transformants Expressing Various Glyphosate-Inactivating Enzymes

In Example 2, the *hph* gene was demonstrated to be an extremely effective selectable marker gene for recovery of glyphosate-resistant plastid transformants in tobacco NT1 cells. Since the HPH enzyme has been shown to phosphorylate glyphosate *in vitro*, this is the most likely mechanism for the observed herbicide resistance. A second gene, the *glpA* gene from *Pseudomonas pseudomallei*, shares extensive amino acid and DNA sequence homology with *hph*. Like HPH, the *glpA* gene product has been demonstrated to possess phosphotransferase activity using either glyphosate or hygromycin B as a substrate. Taken together, these observations suggested to us that *glpA*, when expressed in the plastid like *hph*, would also provide glyphosate resistance to the transformed cells.

To achieve higher levels of glyphosate resistance, it may be desirable to introduce into the plastid organelle enzymes that are capable of degrading glyphosate. One such candidate gene, the *glpB* gene from *Pseudomonas pseudomallei*, is thought to encode a glyphosate-degrading enzyme. Accordingly, we have constructed a plastid expression cassette that co-expresses the *hph* and *glpB* genes together and have introduced this cassette into NT1 cells for the recovery of glyphosate-resistant tobacco NT1 cell plastid transformants. Also, bombardment of regenerable, photosynthetically-active tobacco callus with the *glpB-hph* genes has resulted in the recovery of glyphosate-resistant calli that contain HPH phosphotransferase activity.

Materials and Methods for Example 3

Plasmid construction. Plasmid pSCO2 contains the *hph* and *aadA* genes under control of the petunia 16S rDNA promoter. The *glpB* gene, with its own RBS element

from the *rbcL* gene, was inserted adjacent to and upstream of the *hph* gene in plasmid pSCO2 to create plasmid pSCO3. Thus, a polycistronic transcript would be predicted to be synthesized in the plastid that included the *glpB-hph-aadA* genes. This cassette is embedded within the 3.3 kb *Bam* HI petunia chloroplast DNA fragment found in pSAN307 for targeting into the tobacco plastid chromosome.

Plant cell transformation. Plant cell transformation was carried out as described in Example 2.

Phosphotransferase assays. HPH phosphotransferase assays were carried out as described in Example 2.

Results and Discussion

One of the many attractive features of the non-selective herbicide glyphosate is its rapid degradation by soil microorganisms. In 1995, Peñaloza-Vazquez and colleagues (Peñaloza-Vazquez I) described the isolation of a glyphosate-degrading bacterial strain, *Pseudomonas pseudomallei* II, from glyphosate-treated soil. They further described the cloning and characterization of two genes, *glpA* and *glpB*, which were involved in the degradation of glyphosate. The *glpA* deduced amino acid sequence revealed a significant level of identity to the *E. coli hph* gene, suggesting that *glpA* encoded a phosphotransferase enzyme. This prediction was realized when they demonstrated that the *glpA* enzyme could utilize both glyphosate and hygromycin B as a substrate for phosphorylation (like the HPH phosphotransferase). The *glpB* DNA and deduced amino acid sequence had no significant homology with any other DNA or protein sequences.

Gene expression studies in *E. coli* revealed that cells harboring *glpA* were able to grow in the presence of 100 μg/ml hygromycin B whereas the host strain was inhibited by a concentration of 50 μg/ml, thus confirming its phosphotransferase activity (Peñaloza-Vazquez I). *E. coli* cells harboring *glpB* alone were able to utilize glyphosate

as the sole phosphorous source, suggesting that *glpB* encodes an enzyme with glyphosate-degrading activity (Peñaloza-Vazquez I). Although the activity of the *glpB* enzyme remains uncertain, the authors speculated that it probably converts glyphosate by cleavage of the N-C bond to a breakdown intermediate, aminomethylphosphonic acid.

With a view toward increasing the level of glyphosate resistance achievable in plant cell plastid transformants, we inserted the *glpB* gene into a chloroplast expression cassette already containing the *hph* and *aadA* genes. The *glpB* gene, supplied with its own RBS based upon the *rbcL* gene, was placed immediately upstream of the *hph* gene in pSCO2 (thus creating pSCO3) such that a polycistronic transcript containing *glpB-hph-aadA* would be synthesized by the plastid RNA polymerase (Fig. 2). This cassette, under the control of the petunia chloroplast 16S DNA promoter, is embedded within the 3.3 kb *Bam* HI petunia chloroplast inverted repeat region found in pSAN307 for targeting in the plastid genome (Fig. 3B).

Tobacco NT1 cells were bombarded with plasmid pSCO3 and plastid transformants selected on medium containing 2 mM glyphosate. Within several weeks, microcalli were observed to be proliferating. After several more weeks, the calli were transferred to fresh medium containing 2 mM glyphosate. No calli were ever observed on plates of non-bombarded cells.

Phosphotransferase assays detected the presence of enzymatically-active HPH protein. Cell-free extracts were prepared from the glyphosate-resistant pSCO3 NT1 transformants and tested for their ability to phosphorylate glyphosate *in vitro*. As can be observed in Fig. 7, high levels of HPH phosphotransferase activity were detected in all three pSCO3 transformants. These values were essentially identical to the ones measured for the pSCO2 NT1 transformants (Fig. 7). These results indicate that *hph* is expressed equally well in pSCO2 and pSCO3 NT1 transformants. Therefore, the position of the

hph gene in the dicistronic and polycistronic operons (see Fig. 2) of pSCO2 and pSCO3, respectively, has little, if any, influence on its expression. Little phosphotransferase activity was detected in the extract prepared from untransformed NT1 cells.

Plasmid pSCO3 was also bombarded into a regenerable, photosynthetically-active tobacco cell suspension for the recovery of glyphosate-resistant calli and plants. Glyphosate-resistant green calli were recovered and three transformants observed to contain levels of HPH phosphotransferase activity similar to those observed for pSCO3 NT1 transformants (Fig. 7). DNA gel blot analysis of these pSCO3 transformants should reveal that the *glpB-hph-aadA* expression cassette has integrated into the chloroplast chromosome at its targeted site (as was observed for the NT1 transformants).

Example 4

Plastid Transformation of Non-Photosynthetic Plant Cells Does Not Require an nep Promoter

Allison *et al.* (*EMBO J.* **15**, 2802 (1996)) recently described the generation of transgenic tobacco plants that were genetically-modified in their plastid chromosomes to delete the *rpoB* subunit of the plastid-encoded RNA polymerase (*pep*) enzyme. The resulting transgenic plants (designated ΔrpoB) were albino and lacked the differentiated, photosynthetically-active chloroplast structure typically found in mature leaves. Gene expression studies of these mutants revealed that the transcript abundance of certain plastid genes was dramatically reduced while the abundance of others was relatively unaffected or even slightly increased. Analysis of the genes whose transcript abundance was relatively unaffected (or increased) revealed that the site of transcription initiation for these genes in ΔrpoB plants differed from that found in wild-type plants. The authors concluded that these novel transcripts were synthesized by a nuclear-encoded RNA polymerase (*nep*) enzyme that is synthesized in the cytosol and imported into the plastid.

The transcript accumulation patterns of a significant number of plastid genes in both wild-type and ΔrpoB plants were analyzed and found to fall into one of three categories: one class of genes which contains both *pep* and *nep* enzyme-mediated transcription initiation sites; a second class that contains a transcription initiation site(s) for the *pep* enzyme only; and a third and final class that contains a transcription initiation site(s) for the *nep* enzyme only (Hajdukiewicz et al., *EMBO J.* 16, 4041 (1997). DNA sequence analysis of the nucleotides surrounding the transcription initiation site(s) for the nuclear-encoded RNA polymerase identified a putative *nep* promoter with the consensus sequence ATAGAATAAA, where transcription begins at one (or more) of the last three A residues (Hajdukiewicz et al., *supra*). This sequence, or one very similar, was found in all but one of the ten examined genes with *nep*-mediated transcripts.

Transcript analysis of the tobacco 16S rDNA gene revealed the presence of transcription initiation sites for both *pep* and *nep* enzymes (Fig. 10) (Allison *et al.*, *supra*). The *pep* initiation sites are used almost exclusively in wild-type plants whereas the *nep* initiation site is used predominantly in the ΔrpoB plants. DNA sequence analysis of the 16S rDNA 5′ region revealed the presence of both the familiar -35/-10 *pep*-associated promoter elements as well as a sequence motif homologous to the consensus sequence for a *nep* promoter (Fig. 10). The authors concluded that the *nep* promoter would be preferentially utilized in non-photosynthetic plant tissues such as meristems and roots, which would contain proplastids and amyloplasts, respectively. This conclusion is supported by earlier observations (Vera and Suguira, *Curr. Genet.* 27, 280 (1995)) that although 16S rRNA transcripts were initiated from both *pep* and *nep* promoters in chloroplasts from green tobacco leaves and in proplastids from non-photosynthetic cultured tobacco cells, *nep*-derived transcripts were more abundant than *pep*-derived transcripts in the proplastids. The authors further speculated that plastid transformation

of non-photosynthetic tissues such as embryogenic or non-embryogenic callus and suspension cultures would require a *nep* promoter for efficient expression of foreign genes like selectable marker genes since the *pep* promoter is not efficiently utilized in the plastids of non-photosynthetic tissues.

Examination of the DNA sequence in the P_{rrn} fragment of our plastid expression cassettes (found in pSAN347, pSCO2 and pSCO3) reveals that while the canonical -35/-10-like elements, which comprise the *pep* promoter, are present, the putative *nep* promoter, which has been identified, is absent (Fig. 10). In the genetic constructs described here, the *nep* promoter has been deleted and replaced in the same location by the ribosome-binding site for translation initiation. DNA sequence comparison in this immediate region reveals no significant homology between the P_{rrn} sequence and the tobacco wild-type 16S promoter fragment. It is important to note that a putative *nep* promoter can be identified by DNA sequence inspection in the chloroplast 16S rDNA genes from mustard, soybean, spinach, and maize in the same relative position as identified for the tobacco 16S rDNA gene (Fig. 10). Therefore, this strongly suggests that the *nep* promoter in the P_{rrn} fragment has been deleted and is not merely lacking sufficient homology to be detected by DNA sequence comparison.

It might seem feasible that the expression of the foreign genes in our transgenic lines is due to read-through transcription from other plastid promoters that lie outside of our expression cassette. However, our transgenes are located and oriented on the tobacco chloroplast chromosome in the same manner as first reported by Zoubenko *et al.*, *supra*, (Figs. 3C and 3D). Those authors analyzed transcripts from the *gusA* gene, either promoterless (pLAA25A) or under control of the tobacco 16S rDNA promoter (pLAA24A), situated in the same location and orientation on the tobacco chloroplast chromosome as our cassettes. No *gusA* transcripts were detected in tobacco chloroplasts

containing the promoterless *gusA* gene whereas a highly abundant *gusA* transcript derived from the 16S promoter was found in pLAA24A transformants. Taken together, these results indicate that a functional promoter must be included in an expression cassette to obtain detectable levels of gene expression when the cassette is situated in this location and orientation within the plastid chromosome. Since the tobacco and petunia genomes are extremely highly conserved in both DNA sequence and gene arrangement in this region of the chromosome, the same general observations made in tobacco should apply to petunia.

Based upon the report of Zoubenko *et al., supra*, in conjunction with our own results, which indicate high levels of reporter gene product and HPH phosphotransferase activity in our pSAN347 and pSCO2/pSCO3 plastid transformants, respectively, the inescapable conclusion is that transcription initiation must be directed by the *pep* promoter in our P_{rrn} fragment. The observations of Vera and Suguira, *supra*, who identified 16S rRNA transcripts originating from the *pep* promoter in non-photosynthetic proplastids of cultured tobacco cells, also support this argument. These results strongly indicate that the *nep* promoter identified by Maliga and colleagues is not required for high-level plastid gene expression in plastid transformants of non-photosynthetic plant cells.

Example 5

Plastid Expression Vectors for the Recovery of Monocot Plant Cell Plastid Transformants

All of the major crops grown in the United States as well as abroad, such as wheat, corn, oats, sorghum and rice, are classified as monocotyledonous plants. If the capability to reliably transform the plastid genome was expanded to include these agronomically-important crops and other valuable monocot species (like turfgrass), new

opportunities in crop improvement could be realized. Although the gene content and gene arrangement of plastid genomes is generally very conserved among vascular land plants, there are some significant differences that have been reported. These differences may include genome size, gene content, gene organization, variable spacing between genes, and differing sizes of the single-copy and inverted repeat regions. Therefore, the selection of an insertion site for foreign genes and the associated flanking sequences that surround the insertion site to provide the necessary homology for integration into the plastid chromosome must be carefully considered. The insertion of transgenes into the plastid genome must not disrupt essential chloroplast genes nor seriously interfere with the expression of neighboring genes.

The entire chloroplast genomes of the monocots, *Oryza sativa* (rice) (Hiratsuka *et al.*, *Mol. Gen. Genet.* **217**, 185 (1989)) and *Zea mays* (maize) (Maier *et al.*, *J. Mol. Biol.* **251**, 614 (1995)) have been sequenced. DNA sequence comparison has revealed that these monocot genomes share a very high degree of homology with each other, and to a large extent, with the tobacco plastid genome, the first dicot chloroplast genome to be sequenced in its entirety (Shinozaki *et al.*, *EMBO J.* **5**, 2043 (1986)). However, gene content differences and structural changes were noted between the monocots and the dicot. With these differences noted, we sought to identify an insertion site within the monocot plastid chromosome that would likely be conserved and thus be applicable to a broad range of monocot plant species. Since the inverted repeat region of the dicot plastid genome has already proven to be an excellent site for the targeting of transgenes into the plastid chromosome, this area was selected for further consideration. Moreover, selected portions of the inverted repeat segments of dicot and monocot plastid chromosomes are some of the most highly conserved regions of the plastid genome, and are likely candidates for the identification of a suitable insertion site.

Inspection of the rice and maize plastid inverted repeat sequences revealed the shared presence of an intergenic region that contained no detectable protein-coding regions. Fig. 11 depicts this span, nearly 1 kb in length, located between exon 2 of the *rps12* gene and a putative protein-coding region of unknown function, designated ORF 72. Importantly, the DNA sequence homology in this intergenic region and in the flanking regions was extremely high between maize and rice, suggesting to us that the flanking regions would be suitable for targeting foreign genes (through homologous recombination) into a variety of monocot plastid chromosomes. Therefore, this region appeared to meet the criteria sought for the integration of foreign genes into monocot plastid chromosomes. An additional attractive feature of this region is that, in the unlikely event that DNA integration disrupted an essential chloroplast gene (identified or not), an intact duplicate of this region would still remain on the other copy of the inverted repeat. In this scenario, it would be expected that plastid transformants would be recovered that would contain dissimilar inverted repeat regions.

Since we maintained a primary interest in transforming the plastid genomes of turfgrasses, a 3.1 kb fragment spanning from the *rps7* gene to the *trnV* gene of the inverted repeat region was cloned from the plastid genome of *Agrostis stolonifera*, or bentgrass. Although the bentgrass genome has not yet been sequenced, bentgrass chloroplast structural DNA information is given in Katayama *et al.*, *Curr. Genet.* **29**, 572 (1996). Partial DNA sequence analysis of this fragment revealed that the DNA sequence homology between bentgrass and rice easily exceeded 95%. From the sequence analysis, a unique *Xba* I site within the intergenic region (Fig. 11) was selected as the insertion site for the transgenes. The nearest protein-coding region to this *Xba* I site, ORF72, is nearly 200 bp away; in the opposite direction, the *rps12* gene lies almost 800 bp away. The plastid expression cassettes would then be flanked by ~1 kb of bentgrass plastid sequence

on the side of the ORF72/ORF85 genes and by ~2.1 kb on the side of the *rps12/rps7* genes (Fig. 11) to facilitate homologous recombination events with the resident plastid chromosomes. It is especially worth noting that this same *Xba* I site is also conserved in both the rice and maize plastid genomes.

The 2.2 - 3.2 kb plastid expression cassettes found in plasmids pSAN347, pSCO2 and pSCO3, extending from the P_{rrn} fragment to the T_{psbA} fragment (Fig. 2), were liberated from the petunia chloroplast sequences by digestion with *Not* I and *Pst* I. In the cases of pSCO2 and pSCO3, partial *Pst* I digests were necessary since the *hph* gene contains a *Pst* I site within its coding region. DNA sequence comparison of the petunia chloroplast 16S rDNA promoter and the *rbcL* RBS element in the P_{rrn} fragment to the maize sequences revealed a very high degree of homology, strongly suggesting to us that these regulatory elements would function properly in monocot plastids. The same conclusion was also reached for the tobacco T_{psbA} element, which is required for transcript 3′ end maturation and stability. The *reporter-aadA* (from pSAN347), *hph-aadA* (from pSCO2) and *glpB-hph-aadA* (from pSCO3) cassettes were each inserted into the *Xba* I site of the bentgrass plastid fragment in plasmid pSCO5. All plastid expression cassettes were inserted in both possible directions of transcription (Fig. 11) in the unlikely event that orientation within the inverted repeat would impact the recovery of spectinomycin- and glyphosate-resistant plastid transformants.

Virtually all regeneration systems for monocot plants rely upon the initiation and maintenance of regenerable, non-photosynthetic callus or cell suspension cultures. We chose to test our gene expression vectors for monocot plastid transformation in two monocots, maize and creeping bentgrass. Maize Black Mexican Sweet (BMS) cells, a non-regenerable corn line, provide an extremely attractive target for biolistic transformation (the cell suspensions are very fine and grow well). We bombarded six

plasmids into maize BMS cells for the recovery of glyphosate-resistant (pSCO6 - pSCO9) and spectinomycin-resistant (pSCO10/pSCO11) plastid transformants. In addition, we bombarded these same plasmids into a regenerable, embryogenic cell suspension derived from creeping bentgrass. After plastid transformants were recovered as callus, intact plants were regenerated from the transgenic callus, either in the presence (if the plastid transformants have not yet achieved homoplasmy) or absence of selective pressure (when homoplasmy is achieved).

Example 6

Glyphosate Resistance in Tobacco Plastid Transformants Expressing the hph, glpB, and hph-glpB Co-transfected Glyphosate-Inactivating Enzyme

In Example 2, the *hph* gene was demonstrated to be an extremely effective selectable marker gene for recovery of glyphosate-resistant plastid transformants in tobacco NT1 cells. Since the HPH enzyme has been shown to phosphorylate glyphosate *in vitro*, this is the most likely mechanism for the observed herbicide resistance. A second gene, the *glpA* gene from *Pseudomonas pseudomallei*, shares extensive amino acid and DNA sequence homology with *hph*. Like HPH, the *glpA* gene product has been demonstrated to possess phosphotransferase activity using either glyphosate or hygromycin B as a substrate. We concluded that *glpA*, when expressed in the plastid like *hph*, should also provide glyphosate resistance to the transformed cells.

It was also of interest to investigate other genes that, when expressed in the plastid, might confer glyphosate resistance through alternative (*i.e.*, non-phosphorylating) molecular mechanisms. One such candidate gene, the *glpB* gene from *Pseudomonas pseudomallei*, is thought to encode a glyphosate-degrading enzyme that works in concert with the *glpA* phosphotransferase to confer glyphosate resistance in that microorganism. Earlier gene expression studies in *E. coli* suggested that the *glpB* enzyme highly

preferred the phosphorylated form of glyphosate over the unmodified form as a substrate for degradation. To assess the *glpB* gene in plant cells, plastid expression cassettes that express *glpB* alone or co-express *hph* and *glpB* together were first introduced into tobacco NT1 cells. Despite repeated attempts, no plastid transformants expressing *glpB* alone could be recovered after selection on glyphosate-containing medium. NT1 transformants co-expressing *glpB* and *hph* displayed the same glyphosate resistance properties as *hph*-expressing NT1 lines. These results suggested that *glpB* expression in tobacco NT1 plastid transformants appeared to be inconsequential.

Transplastomic tobacco plants expressing *glpB-hph* were recovered and tested for their glyphosate resistance phenotype. In spray tests conducted in a growth chamber environment, plants expressing *hph* survived ROUNDUP® application rates up to 0.8 kg/Ha. However, *glpB-hph* plants survived a ROUNDUP® application rate of 1.2 kg/Ha, the highest concentration tested was 1.8 kg/Ha. Untransformed control plants died when exposed to only 0.12 kg/ha of glyphosate. Taken together, these results demonstrated that significant levels of glyphosate resistance could be achieved in *hph*-expressing transplastomic plants. Moreover, these results indicate that glyphosate resistance can be augmented by co-expression of the *glpB* gene. Finally, emerging results from experiments focused on achieving monocot plant plastid transformation also indicate that co-expression of *glpB* with *hph* will confer an advantage to plastid transformants selected on glyphosate-containing medium over transformant expressing *hph* only. Overall, these results demonstrate that co-expression of *glpB* with *hph* favorably impacts the glyphosate resistance phenotype achieved in some plastid transformants.

To determine whether the HPH protein exerted its influence solely in the plastid or was also transforted out of the plastid into the cytoplasm, tobacco plants whose plastids were transformed with the *hph* or *hph/glpB* and control tobacco plants nuclear

transformed with hph under control of the 35S promoter were subjected to 30 μg/ml hygromycin. After 15 days of incubation, the chloroplast-transformed plants died under hygromycin selection, but the nuclear transformed plants were still growing. These results suggest that the hygromycin phosphotransferase protein is not exported out of the chloroplasts.

Materials and Methods for Example 6

Plasmid construction. Plasmid pSAN325 contains the *aadA* gene under control of the petunia plastid 16S rDNA promoter. This plasmid was digested with restriction enzymes *Cla* I and *Stu* I, which cleave between the *rrn* promoter and the *aadA* gene. A *Cla* I - *Sma* I restriction fragment containing the *glpB* coding region, supplied with its own synthetic RBS element modeled after the *rbcL* gene, was generated by PCR amplification. This *glpB* gene was then inserted between the *Cla* I and *Stu* I sites of pSAN325 to create plasmid pSCO1. It was predicted that a dicistronic *glpB-aadA* transcript should be synthesized in the plastid.

Plasmid pSCO2 contains the *hph* and *aadA* genes under control of the petunia plastid 16S rDNA promoter. Plasmid pSCO2 was linearized by digestion with *Cla* I, which cleaves just prior to the RBS element of the *hph* gene. The *Cla* I ends were then filled in by DNA synthesis using Klenow DNA polymerase. The same *Cla* I-*Sma* I *glpB* gene used in the construction of pSCO1 was also treated with Klenow DNA polymerase to create a blunt-ended fragment. This *glpB* gene was then inserted in the correct orientation at the modified *Cla* I site adjacent to the *hph* gene in plasmid pSCO2 to create plasmid pSCO3. Thus, a polycistronic transcript would be predicted to be synthesized in the plastid that would include the *glpB-hph-aadA* genes.

The plastid expression cassettes in both pSCO1 and pSCO3 were embedded (at the *Hinc* II site located at the end of ORF70B) within the 3.3 kb *Bam* HI petunia chloroplast DNA fragment found in pSAN307 for targeting into the tobacco plastid chromosome. In both plasmids, the direction of transcription is toward the *rps12* gene.

Plant cell transformation. Plant cell transformation was carried out as described in Example 2.

Phosphotransferase assays. HPH phosphotransferase assays were carried out as described in Example 2.

DNA gel blot analysis. DNA gel blot analysis was carried out as described in Example 1.

Results and Discussion

A two-fold approach was adopted to investigate *glpB* gene expression in plastids. First, with a view toward increasing the level of glyphosate resistance achievable in plant cell plastid transformants, the *glpB* gene was inserted into a chloroplast expression cassette already containing the *hph* (and *aadA*) genes. The *glpB* gene, supplied with its own RBS based upon the *rbcL* gene, was placed immediately upstream of the *hph* gene in pSCO2 (thus creating pSCO3) such that a polycistronic transcript containing *glpB-hph-aadA* would be synthesized by the plastid RNA polymerase (Fig. 2). We hypothesized that the *hph* enzymewould act in a similar manner to the *glpA* protein and phosphorylate glyphosate for immediate breakdown by the *glpB* enzyme.

Second, to assess the level of glyphosate resistance conferred by *glpB* alone, the *glpB* gene was also inserted into a chloroplast expression cassette containing the *aadA* gene, but not the *hph* gene. The same *glpB* gene as found in pSCO3 was placed immediately upstream of the *aadA* gene (thus creating pSCO1) such that a dicistronic

transcript containing *glpB-aadA* would be synthesized by the plastid RNA polymerase (Fig. 2). These expression cassettes, under the control of the petunia chloroplast *rrn* promoter, were embedded (at the *Hinc* II site located at the end of ORF70B) within the 3.3 kb *Bam* HI petunia chloroplast inverted repeat region found in pSAN307 for targeting in the plastid genome (Fig. 3B).

Tobacco NT1 cells were bombarded with plasmids pSCO3 (*glpB-hph-aadA*) and pSCO1 (*glpB-aadA*), and plastid transformants selected on medium containing 2 mM glyphosate. Within several weeks, micro-calli were observed to be proliferating on plates that had been bombarded with pSCO3 DNA, but no micro-calli were observed on plates bombarded with pSCO1. After several additional weeks, glyphosate-resistant, pSCO3-bombarded NT1 calli were transferred to fresh medium containing 2 mM glyphosate. Still, no calli were observed on the plates of pSCO1-bombarded cells. Additional tobacco NT1 cell bombardments were performed with pSCO1 DNA, but no glyphosate-resistant calli were ever recovered. The inability to recover pSCO1 NT1 transformants strongly supported the conclusion from earlier *E. coli* studies that suggested that non-phosphorylated glyphosate was a relatively poor substrate for utilization by the *glpB* protein. Therefore, we focused our attention on whether co-expression of *glpB* with *hph* would increase glyphosate resistance levels in NT1 transformants relative to transformants expressing *hph* alone.

Cell-free extracts were prepared from the glyphosate-resistant pSCO3 NT1 transformants and tested for their ability to phosphorylate glyphosate *in vitro*. As can be observed in Fig. 7, high levels of HPH phosphotransferase activity were detected in all three pSCO3 transformants. These values were very similar to the ones measured for the pSCO2 NT1 transformants (Fig. 7). These results indicate that *hph* is expressed equally well in pSCO2 and pSCO3 NT1 transformants. Therefore, the position of the *hph* gene

in the dicistronic and polycistronic operons (see Fig. 2) of pSCO2 and pSCO3, respectively, has little, if any, influence on its expression. Once again, little phosphotransferase activity was detected in the extract prepared from untransformed NT1 cells.

Since HPH phosphotransferase levels were so similar in pSCO2 (*hph*) and pSCO3 (*glpB-hph*) transformants, we sought to determine if *glpB* expression increased glyphosate resistance levels. Tobacco NT1 plastid transformants expressing *hph* or *glpB-hph* were maintained in 1 mM glyphosate-containing liquid medium. These cells were then used to inoculate fresh liquid cultures containing either 1, 5, 10 or 20 mM glyphosate, and growth was monitored for nearly three weeks. Untransformed NT1 cells were also tested and failed to grow in medium containing the lowest level of glyphosate (1 mM). Both pSCO2- and pSCO3-transformed NT1 cells grew at essentially the same rates in medium containing up to 10 mM glyphosate, the highest level of glyphosate in which growth was observed. No significant cell growth was observed in medium containing 20 mM glyphosate. Therefore, this study could not detect differences in either growth rate in glyphosate-containing medium nor absolute level of glyphosate resistance between *hph* and *glpB-hph*-expressing NT1 cells, suggesting that *glpB* contributed little, if any, to the glyphosate resistance phenotype observed here.

Although phenotypic differences between pSCO2 and pSCO3 NT1 transformants could not be discerned, we wanted to determine if similar results would be obtained in tobacco plants expressing with these same transgenes. To recover transplastomic tobacco plants, plasmids pSCO2 and pSCO3 were bombarded into regenerable, photosynthetically-active tobacco callus. Glyphosate-resistant green calli were recovered for each DNA and were shown to express HPH phosphotransferase activity (Fig. 7). Additionally, DNA gel blot analysis of the pSCO2 and pSCO3 transformants revealed

that the P_{rrn}-*hph-aadA*-T_{psbA} and P_{rrn}-*glpB-hph-aadA*-T_{psbA} expression cassettes, respectively, had integrated into the chloroplast chromosome at the expected targeted site (Fig. 13).

Tobacco shoots were regenerated from the transformed calli in the presence of glyphosate and assayed for HPH phosphotransferase activity. As can be observed in Figure 12, leaf extracts prepared from *in vitro*-maintained pSCO2 and pSCO3 transplastomic plants contained similarly high levels of HPH phosphotransferase activity. The tobacco plants were eventually moved to the growth chamber for assessment of glyphosate resistance levels. Transplastomic tobacco plants expressing *hph* alone or co-expressing *glpB-hph* were sprayed with commercial formulations of ROUNDUP® at rates up to 1.8 kg/Ha (equivalent to 72 oz./acre) (Fig. 14). It was observed that pSCO2 (*hph*) plants exhibited no glyphosate-related symptoms at 0.8 kg/Ha but that damage was observed when sprayed at a rate of 1.2 kg/Ha. This was ~10-fold above the level that was required to kill untransformed tobacco plants. When pSCO3 (*glpB-hph*) plants were sprayed, no herbicide-related damage was observed at a rate of 1.2 kg/Ha, the same rate that had affected pSCO2 plants. When the ROUNDUP® application rate was increased to 1.8 kg/Ha, the highest concentration tested, glyphosate-related damage was observed on the pSCO3 plants. This increased level of glyphosate resistance in pSCO3 plants was reproducible and most likely can be attributed to *glpB* expression within the plastid.

To explain these results, it could be imagined that the HPH phosphotransferase first modifies the glyphosate molecules entering the plastid in both pSCO2 and pSCO3 plants, thus deactivating the herbicide. This enzymatic step constitutes the primary mode of action for the observed glyphosate resistance phenotype in pSCO2 and pSCO3 tobacco plants. However, the possibility that the phosphorylated glyphosate molecule still retains a residual amount of binding activity for the EPSPS enzyme cannot be ruled out. Thus,

in the absence of the *glpB* enzyme, the phosphorylated glyphosate may still exert some inhibitory activity within the plastid compartment. However the presence of the *glpB* protein, with its glyphosate-degrading activity, should reduce intracellular concentrations of the modified glyphosate, effectively increasing the overall level of glyphosate resistance.

Alternatively, it could be postulated that phosphate moiety in glyphosate is cleaved by random phosphatases within the plant cell and/ or perhaps non-enzymatic hydrolyzation, thereby restoring full EPSPS-binding activity. However, in the presence of the *glpB* protein, the phosphorylated glyphosate molecule would be immediately degraded, thereby making it unavailable to intracellular phosphatases.

In addition to these observations in tobacco plants, we have noted that *glpB* expression might be providing enhanced glyphosate resistance to maize calli co-expressing *hph* versus *hph* alone. We have casually noted that during selection of bombarded maize suspension cells on glyphosate-containing medium, plates of cells bombarded with a plasmid designed to co-express both genes in the plastid exhibit both faster-growing and higher numbers of transformants than plates bombarded with *hph* alone. This same observation has also been noted after similar bombardments of rice suspension cells. Taken together with the ROUNDUP® spray tests of the transplastomic tobacco plants, these observations indicate that co-expression of *glpB-hph* in the plastid compartment can improve the glyphosate resistance phenotype.

At this time, we are uncertain as to why no phenotypic differences were observed in tobacco NT1 pSCO2 and pSCO3 transformants (as compared to the whole plants). It may be that such differences are dependent upon plant species (tobacco vs. maize) and/or tissue type (callus vs. plant). Finally, it should be noted that a plastid expression cassette that contains the *hph* gene under regulatory control of the *rrn* promoter (see PSCO35 in

Fig. 2) has also yielded glyphosate-resistant tobacco transformants (as verified by detection of an *hph*-specific PCR product). This indicates that the *aada* gene, which is found in plasmids pSCO2 and pSCO3, is dispensable for the recovery of glyphosate-resistant transformants.

The glyphosate-resistant tobacco plants were grown to flowering. The plants appeared phenotypically normal, and were as vigorous as the control plants. These plants were then selfed and crossed to wild-type plants. the transgenic plants appeared to be fully male and female fertile. Seed was collected from the crosses and was screened for the presence of the *aadA* gene by germination in the presence of spectinomycin (seedlings are green if the gene is present in the chloroplast, white if the gene is absent). When the transgenic plants were used as the female parent, all of the progeny were green, but when the wild-type parent was pollinated by the transgenics, all progeny were white, proving classical maternal inheritance, as expected.

Example 7

Recovery of Glyphosate-Resistant Plant Cell Plastid Transformants from the Maize and Bentgrass Monocots

As described in Examples 2, 3 and 6, transplastomic tobacco NT1 cultured cells and plants were recovered using the *hph* gene (and, in some cases, *glpB*) to confer resistance to the herbicide, glyphosate. With a view toward expanding the application of this plastid transformation technology beyond tobacco, the agronomically-important monocot crop plants were targeted for plastid genome manipulation.

An *hph* expression cassette, under the control of the strong, constitutive petunia 16S rDNA promoter, was embedded within a segment of the creeping bentgrass chloroplast inverted repeat region to provide DNA sequence homology for recombination events with the resident monocot plastid chromosomes. After bombardment of non-

regenerable maize suspension cells, glyphosate-resistant calli were recovered in modest numbers. High levels of HPH phosphotransferase activity were detected in the glyphosate-resistant maize transformants. DNA gel blot analysis confirmed that the introduced *hph* gene had integrated into the maize plastid chromosome at the expected site by homologous recombination. Bombardment of a regenerable creeping bentgrass cell suspension also resulted in the recovery of glyphosate-resistant calli. A bentgrass plant regenerated from one of the glyphosate-resistant calli exhibited a high level of glyphosate resistance after spray application of the herbicide ROUNDUP®. Bombardment of a regenerable rice cell suspension has also resulted in the recovery of glyphosate-resistant calli; similar transformation experiments have recently been undertaken in wheat, too. These results provide strong, convincing evidence that the plastid-expressed *hph* gene, when used as a selectable marker gene together with glyphosate as the selective agent, permits relatively facile manipulation of the plastid genomes of monocotyledonous plants.

The novel genetic constructs described herein have extended the range of land plant species in which plastid transformation is feasible and may be used to recover glyphosate-resistant plants with commercially-acceptable levels of herbicide resistance.

Materials and Methods for Example 7

Plasmid construction. Oligonucleotide primers were designed to anneal to sequences found in the *trnV* and *rps7* genes of the creeping bentgrass chloroplast inverted repeat region. A 3.1 kb fragment spanning from *trnV* to *rps7* was amplified by PCR and digested with *Sac* I, which cleaves at a primer-specific site. This fragment, which will provide the flanking DNA sequences necessary for facilitating integration of transgenes

into monocot plastid genomes, was inserted into *Sac* I-digested pGEM5 DNA (Promega) to create plasmid pSCO5.

The 2.2 - 3.2 kb plastid expression cassettes found in plasmids pSAN347, pSCO2 and pSCO3, extending from the P_{rrn} fragment to the T_{psbA} fragment (Fig. 2), were liberated from the petunia chloroplast sequences by digestion with *Not* I and *Pst* I. In the cases of pSCO2 and pSCO3, partial *Pst* I digests were necessary since the *hph* gene contains a *Pst* I site within its coding region. All restriction fragments were treated with T4 DNA polymerase to create blunt-ended restriction fragments. Plasmid pSCO5 was linearized by digestion with *Xba* I, which cleaves in the intergenic region between the *trnV* and *rps7* genes, and the ends filled in by DNA synthesis using T4 DNA polymerase. The P_{rrn}-*reporter-aadA*-T_{psbA} (from pSAN347), P_{rrn}-*hph-aadA*-T_{psbA} (from pSCO2) and P_{rrn}-*glpB-hph-aadA*-T_{psbA} (from pSCO3) cassettes were each inserted into the now-modified *Xba* I site of the bentgrass plastid fragment in plasmid pSCO5. All plastid expression cassettes were inserted in both possible directions of transcription (Fig. 11) in the unlikely event that orientation within the inverted repeat would impact the recovery of spectinomycin- and glyphosate-resistant plastid transformants.

Plant cell transformation. Plant cell transformation was carried out as essentially as described in Example 2.

Phosphotransferase assays. HPH phosphotransferase assays were carried out as described in Example 2.

DNA gel blot analysis. DNA gel blot analysis was carried out as described in Example 1.

Results and Discussion

Since we maintained a primary interest in transforming the plastid genomes of turfgrasses, a 3.1 kb fragment spanning from the *rps7* gene to the *trnV* gene of the inverted repeat region was cloned from the plastid genome of *Agrostis stolonifera*, or bentgrass. Partial DNA sequence analysis of this fragment revealed that the DNA sequence homology between bentgrass and rice easily exceeded 95%. From the sequence analysis, a unique *Xba* I site within the intergenic region (Fig. 11) was selected as the insertion site for the transgenes. The nearest protein-coding region to this *Xba* I site, ORF72, is nearly 200 bp away; in the opposite direction, the *rps12* gene lies almost 800 bp away. The plastid expression cassettes would then be flanked by ~1 kb of bentgrass plastid sequence on the side of the ORF72/ORF85 genes and by ~2.1 kb on the side of the rps12/*rps7* genes (Fig. 11) to facilitate homologous recombination events with the resident plastid chromosomes. It is especially worth noting that this same *Xba* I site is also conserved in both the rice and maize plastid genomes.

The P_{rrn}-*reporter-aadA*-T_{psbA} (from pSAN347), P_{rrn}-*hph-aadA*-T_{psbA} (from pSCO2) and P_{rrn}-*glpB-hph-aadA*-T_{psbA} (from pSCO3) cassettes were each inserted into this *Xba* I site of the bentgrass plastid fragment in plasmid pSCO5. All plastid expression cassettes were inserted in both possible directions of transcription (Fig. 11) in the unlikely event that orientation within the inverted repeat would impact the recovery of spectinomycin- and glyphosate-resistant plastid transformants. In addition, there was considerable concern that the dicot regulatory elements employed in these cassettes (petunia *rrn* promoter and tobacco *psbA* 3′-flanking region) might not be fully functional in monocot plastids. DNA sequence comparison of the petunia chloroplast 16S rDNA promoter and the *rbcL* RBS element in the P_{rrn} fragment to the maize sequences revealed a very high degree of homology, strongly suggesting to us that these *cis*-acting regulatory elements should indeed function properly in monocot plastids. The same conclusion was

also reached for the tobacco T_{psbA} element, which is required for transcript 3′ end maturation and stability, although any other element that provides 3′-end maturation and stability can be used in place of T_{psbA}. For example, elements having stem-loop structures can also be used for transcript 3′ end maturation and stability.

Virtually all the regeneration systems that have been established for monocot plants rely upon the initiation and maintenance of regenerable, non-photosynthetic callus or cell suspension cultures. We chose to test our monocot plastid transformation vectors in three monocot species, maize, rice and creeping bentgrass. Maize Black Mexican Sweet (BMS) cells, a non-regenerable tissue culture line, provide an extremely attractive target for biolistic transformation (the cell suspensions are very fine and grow well). Initially, plasmids pSCO10 and pSCO11 (P_{rrn}-*reporter-aadA*-T_{psbA}) were bombarded into maize BMS cells. Two days after bombardment, cells were assayed for reporter gene product activity by incubation in the presence of substrate-containing buffer. Microscopic examination of the cells revealed a number of extremely small foci manifesting reporter gene expression that were not observed on plates of cells bombarded with non-reporter gene-containing plasmid DNA (data not shown). Moreover, the pSCO10/pSCO11-bombarded cells were distinctively different from other cells that that were bombarded with plasmid DNA containing the reporter gene fused to a nuclear promoter (which were larger and more diffuse). These results supported our prediction that the dicot regulatory elements employed here in our plastid expression cassettes would be functional in maize (and other monocot) plastids.

To continue, plasmids pSCO6/pSCO7 (P_{rrn}-*hph-aadA*-T_{psbA}) and pSCO8/pSCO9 (P_{rrn}-*glpB-hph-aadA*-T_{psbA}) were bombarded into maize BMS cells. After bombardment, the cells were moved to selective medium containing 2 mM glyphosate for nearly two months. After this period of selection, glyphosate-resistant calli were recovered.

Initially, HPH phosphotransferase assays were carried out to detect *hph* gene expression in pSCO6 transformants. Cell-free extracts were prepared from the glyphosate-resistant pSCO6 calli and tested for their ability to phosphorylate glyphosate *in vitro*. As can be observed in Fig. 15, high levels of HPH phosphotransferase activity were detected in the BMS pSCO6 transformants. These amounts are comparable to or higher than the HPH phosphotransferase levels found in tobacco NT1 pSCO2 transformants expressing the same *hph* expression cassette. Little phosphotransferase activity was detected in the extract prepared from untransformed BMS cells.

DNA gel blot analysis was carried out to determine if the *hph-aadA* cassette had inserted into the maize plastid genome. Total cellular DNA was isolated, digested with *Bam* HI, and probed with radiolabeled *hph* DNA. As can be observed in Fig. 16, the expected 5.4 kb *Bam* HI fragment was observed in all six glyphosate-resistant cell lines. The copy number of this fragment was measured to be approximately 500-1,000 copies per cell. If the *hph-aadA* cassette has inserted into the expected chromosomal location, the wild-type 3.2 kb *Bam* HI fragment should be replaced by a larger, novel 5.4 kb *Bam* HI fragment when probed with bentgrass chloroplast DNA from plasmid pSCO5 (Fig. 3D). In DNA from untransformed BMS cells, the expected 3.2 kb *Bam* HI fragment was detected (Fig. 16). However, in all seven glyphosate-resistant lines, the anticipated 5.4 kb *Bam* HI fragment was detected, indicating correct integration at the expected chromosomal location. No wild-type 3.2 kb *Bam* HI fragment was detected in any of the seven lines.

The lack of wild-type fragments illustrates two important points. First, the *hph-aadA* cassette has been "copy-corrected" from one copy of the inverted repeat to the other inverted repeat. Second, all the chloroplast chromosomes have been transformed, indicating that homoplasmy has been achieved in each of the six lines examined. Based

upon our observations in tobacco and the turfgrass studies presented below, one would expect that if these glyphosate-resistant calli were capable of regeneration the resulting transgenic corn plants would be highly resistant to spray applications of glyphosate (see turfgrass studies below for support of this statement).

In addition, some of these same plasmids (pSCO6 and pSCO9) were bombarded into a regenerable, embryogenic cell suspension derived from creeping bentgrass. A small number of glyphosate-resistant bentgrass calli were recovered after selection on medium containing up to 3 mM glyphosate. PCR analysis was carried out to determine if the glyphosate-resistant calli contained the *hph* gene. As can be observed in Figure 17, the expected 0.8 kb *hph* PCR fragment was observed in each of the four glyphosate-resistant calli. No PCR product was observed in genomic DNA prepared from untransformed callus.

Shoots were regenerated from one of the glyphosate-resistant calli and, after rooting, transferred to the greenhouse. After acclimation, the transgenic bentgrass plant and a untransformed plant were sprayed with a commercial formulation of ROUNDUP® herbicide at a rate equal to 1.2 kg/Ha. As dramatically shown in Figure 18, the transgenic bentgrass plant showed no signs of any glyphosate-related damage and continued to grow normally while the control plant succumbed. This result is entirely consistent with the strong glyphosate resistance phenotype that we had previously observed in transplastomic tobacco plants expressing the same *hph* expression cassette. This result thus extends the glyphosate resistance phenotype conferred by plastid-expressed *hph* genes to a horticulturally-important monocotyledonous plant species.

We also introduced the pSCO6-pSCO9 series of plastid expression vectors into a regenerable rice cell suspension. For these experiments, rice suspension cells were collected onto filter paper, bombarded with plasmid DNA, and selection carried out on

selective medium containing 2 mM glyphosate. Glyphosate-resistant calli were recovered and maintained on medium containing 1 mM glyphosate (where they proliferate faster). Fig. 19 shows that these calli contain an *hph*-specific PCR product.

Finally, bombardment of cultured immature wheat embryos (*cv.* Bobwhite) with plasmids pSCO6-pSCO9 was undertaken to recover glyphosate-resistant plastid transformants of wheat. These plasmids will permit the recovery of transplastomic wheat plants that are highly resistant to applications of ROUNDUP® herbicide.

Example 8

General Utility of hph-based Plastid Expression Cassettes in the Recovery of Glyphosate-Resistant Plastid Transformants in Other Dicotyledonous Plant Species

Successful recovery of glyphosate-resistant maize and rice plastid transformants was facilitated, in part, by the plastid genetic information available for these species(Hiratsuka *et al., supra*, for rice; Maier *et al., supra*, for maize; Katayama *et al.* for bentgrass and the availability of extensive tissue culture methods for the manipulation of these species under *in vitro* conditions.

To provide a more stringent and rigorous test of the general utility of our plastid transformation system, we proposed to transform the plastid genomes of avocado and papaya, two relatively obscure and exotic plants that have not been extensively used in transgenic studies. Of similar importance for these experiments, their plastid genomes are virtually uncharacterized. We have found that glyphosate-resistant avocado cell lines and papaya plants with transformed plastid chromosomes could indeed be recovered with relative ease. The conclusions reached here demonstrate that widespread, routine manipulation of the plastid genomes in a diverse array of land plants is now feasible using the plastid transformation system described herein.

Materials and Methods for Example 8

Plasmid construction. Plasmid pSCO2 has already been described in Example 2.

Plant cell transformation. Plant cell transformation was carried out as essentially as described in Example 2.

Phosphotransferase assays. HPH phosphotransferase assays were carried out as described in Example 2.

DNA gel blot analysis. DNA gel blot analysis was carried out as described in Example 1.

Results and Discussion

As noted previously, the entire chloroplast genomes of several dicots and monocots, including *Nicotiana tabacum* (tobacco) (Shinozaki *et al.*, *supra*), *Arabidopsis thaliana* (unpublished results), *Oryza sativa* (rice) (Hiratsuka *et al.*, *supra*) and *Zea mays* (maize) (Maier *et al.*, *supra*) have been sequenced. DNA sequence comparison has revealed that these plastid genomes generally share a very high degree of homology with each other. However, gene content differences and structural changes were noted between the monocots and the dicots as well as within the two groups. With these differences noted, we sought to identify a highly conserved region within the plastid genome that could be exploited for transformation purposes in a broad range of land plant species. Since the inverted repeat region has been characterized to possess some of the most highly conserved regions of the entire plastid genome and has already proven to be an excellent site for the targeting of transgenes into either dicot or monocot plastid chromosomes, this area was selected for further consideration. The intergenic region between the *trnV* and *rps12* genes has already proven to be a useful site for the

integration of transgenes, and the flanking sequences that surround this region direct efficient integration of the transgenes into the plastid chromosome.

With a view toward assessing the general utility of our existing *hph*-based plastid expression cassettes and glyphosate selection system, we proposed that plasmid pSCO2 be bombarded into regenerable cell cultures of avocado and papaya, two fairly exotic fruit-bearing plant species that have not been used extensively for generation of transgenic plants. Moreover, virtually no chloroplast DNA sequence data exists for these two species; only the DNA sequences of their *rbcL* genes has been deposited in GenBank (version 105.0). Thus without any clear knowledge of the gene content, gene order, or DNA sequence of the chloroplast inverted repeat regions of these plants, and without any prior knowledge of the compatibility of these cell culture systems with glyphosate as a selective agent, we initiated plasmid DNA bombardments. We fully recognized that our proposal to achieve avocado or papaya plastid transformation would represent a serious challenge to our hypothesis that glyphosate selection of a plastid-expressed *hph* gene cassette will readily result in recovery of plastid transformants, even in relatively obscure plants with virtually uncharacterized plastid genomes.

Plasmid pSCO2 was precipitated onto tungsten microparticles and bombarded into avocado and papaya embryogenic cells for selection on either 1 mM or 2 mM glyphosate-containing medium. Within several weeks micro-calli were first observed. Calli continued to proliferate after transfer to fresh selective medium containing 2 mM glyphosate. Papaya shoots were eventually regenerated and whole, rooted papaya plants were recovered.

After the avocado calli and papaya plants had reached an appropriate size, phosphotransferase assays were carried out to detect the enzymatic activity of the HPH protein. Cell-free extracts were prepared from the glyphosate-resistant avocado calli and

tested for their ability to phosphorylate glyphosate *in vitro*. As can be observed in Fig. 20, a wide range of HPH phosphotransferase activities (from moderate to very high) was detected in all five pSCO2 transformants. Little phosphotransferase activity was detected in the extract prepared from untransformed avocado cells. The wide range of HPH phosphotransferase activities may be attributable to varying degrees of heteroplasmy (*i.e.*, lines with high phosphotransferase levels may contain a higher percentage of *hph*-containing plastid chromosomes than lines that display more moderate activity levels). Leaf extracts prepared from two *in vitro*-maintained glyphosate-resistant papaya plants also exhibited high levels of HPH phosphotransferase activity.

DNA gel blot analysis was carried out to determine if the *hph-aadA* cassette had inserted into the avocado and papaya plastid genomes. Total cellular DNA was isolated, digested with *Bam* HI, and probed with radiolabeled *hph* DNA. As can be observed in Fig. 21, the expected 5.5 kb *Bam* HI fragment was observed in all samples from the glyphosate-resistant lines. If the *hph-aadA* cassette has inserted into the expected chromosomal location, the wild-type 3.3 kb *Bam* HI fragment should be replaced by a larger, novel 5.5 kb *Bam* HI fragment when probed with petunia chloroplast DNA from plasmid pSAN307 (Fig. 3D). In DNA from untransformed tissues, the expected 3.3 kb *Bam* HI fragment was detected (Fig. 21). However, in all glyphosate-resistant lines, the anticipated 5.5 kb *Bam* HI fragment was detected, indicating correct integration at the expected chromosomal location. No wild-type 3.3 kb *Bam* HI fragment was detected in any of the seven lines.

The lack of wild-type fragments illustrates two important points. First, the *hph-aadA* cassette has been "copy-corrected" from one copy of the inverted repeat to the other inverted repeat. Second, all the chloroplast chromosomes have been transformed, indicating that homoplasmy has been achieved in each of the seven lines examined.

These studies provide convincing evidence that plastid transformants can be successfully recovered with relative ease without any prior knowledge of the gene content, gene order or gene sequence of the plastid genome in the host plant species. These results further dramatically illustrate the broad utility of an *hph*-based plastid gene expression cassette and glyphosate as an extremely efficient selectable maker gene and selective agent, respectively, for the recovery of transplastomic plant cells. These findings strongly indicate that the plastid genome of any plant species that is amenable to manipulation under tissue culture conditions can now be genetically modified for crop improvement purposes or to address fundamental questions within the context of a basic research program.

Example 9

Recovery of Glyphosate-Resistant Tobacco NT1 Cell Plastid Transformants Expressing glpA

Several independent observations strongly suggested to us that *glpA* might effectively substitute for the *hph* gene in conferring glyphosate resistance to plant cell plastid transformants in the transformation scheme disclosed herein. First, Peñaloza-Vazquez *et al.* (Peñaloza-Vazquez I) reported that the *glpA* protein shared extensive regions of amino acid homology to the *hph* gene product. Second, both the *glpA* and *hph* proteins possess phosphotransferase activities that utilize hygromycin and glyphosate equally well as substrates for phosphorylation *in vitro*. Finally, *glpA* and *hph* were able to confer both hygromycin and glyphosate resistance to *E. coli* cells harboring either of these genes on plasmids. Taken together, *glpA* appeared to us to be an excellent candidate gene for conferring glyphosate resistance in plant cell plastid transformants.

Unanticipated cloning problems presumably associated with *glpA* over-expression in *E. coli* required the construction of complementary *glpA*-containing

vectors. Briefly, two *glpA*-containing plasmids were assembled, neither of which alone could express *glpA* phosphotransferase activity well, if at all, in *E. coli* or plastids. However, if co-bombarded into the plastid, and taking advantage of the active homologous recombination system in this organelle, recombination between the two plasmids could lead to restoration of full and complete *glpA* expression. Indeed, co-bombardment of the two *glpA*-containing plasmids into tobacco NT1 cells yielded glyphosate-resistant plastid transformants that expressed high levels of *glpA* phosphotransferase activity.

Homologous recombination between co-introduced plasmids in the plastid becomes yet another tool for manipulating the plastid genomes of land plants, thereby permitting the introduction and expression of genes in the plastid that otherwise might not be achievable.

Materials and Methods for Example 9

Plasmid construction. The general strategy to recover *glpA*-expressing plastid transformants was to construct two complementary *glpA*-containing plasmids, neither of which alone would express *glpA* in *E. coli* or the plastid, but when recombined within the plastid, would restore *glpA* activity. These two plasmids were designated as containing either *defective* or *corrective glpA* genes, with recovery of glyphosate-resistant transformants dependent upon recombination-mediated 'repair' of the *defective* gene by the *corrective* copy.

To construct the *corrective glpA* version, a 1.3 kb *Bam* HI - *Xba* I *glpA*-containing fragment (coding region, only) was inserted between the *Bam* HI and *Xba* I sites of cloning vector, pUC118. This particular *glpA* gene lacks a plastid-like RBS element that would be recognized and utilized by prokaryotic-like ribosomes; thus

translation initiation at the *glpA* initiator codon should be rare, if at all, in *E. coli* or plastids. At the same time, plasmid pSAN325 was digested with *Stu* I and *Eco* RV to liberate a ~1.8 kb fragment containing, in order, the *aadA* coding region (with its own plastid-like RBS element), T_{psbA} sequences, and ~0.8 kb of flanking petunia chloroplast inverted repeat DNA in the vicinity of the *rps12* gene. This *Stu* I-*Eco* RV fragment was then inserted into the *Hinc* II site of the MCS region of pUC118 so that the *aadA* gene was now adjacent to the *glpA* 3′ end. The resulting plasmid, pSCO18, contains a *glpA*-*aadA*-T_{psbA} cassette flanked at its 3′ end with petunia chloroplast DNA for facilitating DNA integration into the plastid chromosome. This plasmid, when introduced alone into the plastid, would not be expected to confer glyphosate resistance since the *glpA* gene lacks both a plastid promoter and a plastid-like RBS element for efficient transcription and translation, respectively. Moreover, double homologous recombination events between the plasmid and the plastid chromosome leading to integration should occur rarely, if at all, since the gene cassette is flanked on only one side with chloroplast DNA sequences.

To construct the *defective glpA* copy, a unique *Nco* I site within the *glpA* coding region was targeted for mutagenesis. Modification of the *Nco* I site (cleavage followed by DNA synthesis fill-in) would be expected to cause a frameshift mutation leading to the formation of two consecutive in-frame nonsense codons immediately after the destroyed *Nco* I site. The predicted outcome would be the synthesis of a truncated *glpA* protein (lacking nearly 25% of its amino acids) with dramatically reduced or abolished phosphotransferase activity. To accomplish this, a *glpA*-containing plasmid (coding region, only) was linearized by digestion with *Nco* I, treated with Klenow DNA polymerase to fill-in the ends by DNA synthesis, and then re-ligated. Successful destruction of the *Nco* I site was verified by the inability of *Nco* I to digest the resulting

clones. A plastid-like RBS element was then added to the defective *glpA* gene (now designated as **glpA**) for efficient translation in the plastid. The **glpA** gene was liberated from vector sequences by digestion with *Xba* I and treated with Klenow DNA polymerase to yield blunt ends. At the same time, plasmid pSAN325 was digested with *Cla* I and *Stu* I, which cut between the petunia plastid *rrn* promoter and the *aadA* gene to provide a site for insertion of the **glpA** gene. The *Cla* I site was also made blunt-end by the action of Klenow DNA polymerase. The **glpA** gene was then inserted in the correct orientation to yield the plastid gene expression cassette, P_{rrn}-**glpA**-*aadA*-T_{psbA}; the cassette being situated at the end of the ORF70B gene within the inverted repeat region of petunia chloroplast DNA found in pSAN307. The *defective glpA* plasmid was designated pSCO24.

Plant cell transformation. Plant cell transformation was carried out essentially as described in Example 2 except that the bombarded cells on filter paper were transferred to selective medium containing 2 mM glyphosate. Equivalent amounts of pSCO18 and pSCO24 were co-precipitated onto tungsten microparticles for all bombardments.

Phosphotransferase assays. Phosphotransferase assays were carried out as described in Example 2.

DNA gel blot analysis. DNA gel blot analysis was carried out as described in Example 1.

Results and Discussion

To test the hypothesis that *glpA*, like *hph*, could confer glyphosate resistance in plant cell plastid transformants, a *glpA*-containing fragment (coding region, only) was designed that could be conveniently placed under control of the strong, constitutive

petunia plastid *rrn* promoter. However, despite numerous cloning attempts in *E. coli*, no desired clone could be recovered. These failures were especially surprising since the identical cloning strategy had been successfully used for *hph* and *glpB* in the construction of plasmids pSCO2 and pSCO1, respectively. Two possible explanations for these observations were considered: i) the desired transgene was inherently structurally unstable and could not be maintained in *E. coli*; or ii) *glpA* over-expression in *E. coli* (the petunia *rrn* promoter is very active in *E. coli*) was toxic to the cells. To address both these possibilities simultaneously, alternative plastid expression vectors were chosen for insertion of the *glpA* gene. Additionally, further cloning protocols were designed that would permit the *glpA* gene to insert bidirectionally, in either the sense or anti-sense orientation (relative to the *rrn* promoter).

To summarize these exhaustive efforts, clones containing the *glpA* gene in the sense orientation (with respect to *rrn*) could not be recovered; only plasmids in which the *glpA* gene was oriented in the anti-sense orientation were observed. These results strongly suggested that *glpA* over-expression in *E. coli* was problematic. Attempts were made to recover the desired clone in a pACYC184-based plasmid vector, with the hope that the lower plasmid copy number would reduce *glpA* expression levels sufficiently. However, once again, no desired clones were recovered.

The strategy that ultimately proved successful was designed around the idea that if two *glpA*-containing constructs could be separately assembled, neither of which alone could express a fully functional *glpA* protein in *E. coli* or the plastid, then homologous recombination between the two plasmids within the plastid might reconstitute a fully functional *glpA* gene, thus permitting the recovery of glyphosate-resistant plastid transformants. Accordingly, two *glpA*-containing plasmids were constructed to achieve this goal, as shown in Figure 22. The approach followed was to first assemble one

plasmid known as the *defective* copy. This plasmid, designated pSCO24, contains a mutated *glpA* coding region under control of the petunia plastid *rrn* promoter. To create the mutant *glpA* gene (designated **glpA**), a unique *Nco* I site within the coding region was abolished, in the process causing a frameshift mutation. As a result, two consecutive in-frame nonsense codons were created adjacent to the modified site, leading to the predicted synthesis of a truncated **glpA** protein (lacking ~100 amino acid residues, or ~25% of the protein). After the **glpA** gene was created, it was found that it could be placed under control of the *rrn* promoter with ease, suggesting that the truncated **glpA** protein had either dramatically reduced or abolished phosphotransferase activity. A second plasmid (pSCO18), designated as the *corrective* copy, was constructed that contained a promoterless wild-type *glpA* gene also lacking a plastid-like ribosome binding site (Fig. 22). This gene would not be predicted to be expressed well in *E. coli* or the plastid since it lacks both a promoter and a ribosome binding site for efficient transcription and translation initiation, respectively.

The P_{rrn}-**glpA**-*aadA*-T_{psbA} cassette in plasmid pSCO24, the *defective* copy, was flanked on both sides by regions of chloroplast DNA (from the petunia inverted repeat region) to facilitate integration of the transgene into the plastid chromosome. The *corrective* copy was flanked with chloroplast DNA only at its 3′ end and therefore should integrate rarely, if at all, when introduced alone into the plastid chromosome. However, co-introduction of the *defective* and *corrective* copies into the plastid would permit recombination between shared sequences on the plasmids. If homologous recombination between pSCO24 and pSCO18 were to occur within the *glpA* sequences prior to the mutated *Nco* I site and then again beyond the mutation somewhere within the *aadA* gene or flanking chloroplast DNA region, a fully functional *glpA* gene should be restored. The end-products of these double homologous recombination events could be recovered by

their ability to confer glyphosate resistance. It should be noted that recombination between the regions of homology on plasmids pSCO24 and pSCO18 may occur while both DNA's are extra-chromosomal (plasmid-to-plasmid). Alternatively, pSCO24/pSCO18 recombination may proceed after the *defective* **glpA** cassette in pSCO24 has already integrated into the plastid chromosome (plasmid-to-chromosome). Nonetheless, it was predicted that a pair of double homologous recombination events would be required to recover *glpA*-expressing, glyphosate-resistant transformants. However, it should also be noted that *a priori* we could not rule out the possibility that *glpA* over-expression in the plastid might be lethal to the plant cell, as was presumably observed for *E. coli*.

Equivalent amounts of plasmids pSCO24 and pSCO18 DNA were co-precipitated onto tungsten microparticles, co-bombarded into tobacco NT1 cells, and the cells subsequently maintained on selective medium containing 2 mM glyphosate. Within 3 weeks after bombardment, small glyphosate-resistant calli could be observed growing on the selective medium. When the NT1 calli reached an appropriate size, a small sample was removed and cell-free extracts were prepared to measure *glpA* phosphotransferase levels. Indeed, all four extracts contained levels of glyphosate-phosphorylating activity (see Figure 23) that were similar to HPH phosphotransferase levels in pSCO2 and pSCO3 hph-expressing NT1 calli (Fig. 7). Little phosphotransferase activity was observed in extracts prepared from untransformed NT1 cells. DNA gel blot analysis was carried out to determine if the *glpA-aadA* cassette had inserted into the plastid genome. As can be observed in Fig. 24A, radiolabeled *glpA* DNA hybridized to two *glpA*-containing restriction fragments (2.1 kb and 1.0 kb) in DNA prepared from each of four glyphosate-resistant NT1 transformants (lanes 4-7). No hybridization was detected in the lane

containing DNA from untransformed cells (lane 3). When the same transformants were probed with petunia plastid DNA sequences from pSAN307, the expected 3.3 kb BamHI fragment was observed in DNA prepared from untransformed cells (Fig. 24B, lane 3). By contrast, the DNA samples from the glyphosate-restnt NT1 calli exhibited 2.6 kb and 2.1 kb-hybridizing fragments, the expected sizes if integration had occurred at the correct site (Fig. 24B, lanes 4-7. The appearance of two hybridization signals in the glyphosate-resistant transformants is very significant as it demonstrates that the NcoI site which had been deleted in pSCO24 (see Fig. 22) was restored after recombination with the wild-type *glpA* gene found in pSCO18.

The observation that multiple plasmids simultaneously co-bombarded into the plastid will recombine with one another and/or recombine sequentially with the plastid chromosome opens up new avenues for genetic manipulation of this genome. Already, we have demonstrated that co-bombardment of partially homologous plasmids permitted the introduction and expression of transgenes in the plastid that otherwise might not have been achievable. A second potential advantage offered by this technology includes the ability to introduce larger segments of foreign DNA into the plastid chromosome. At present, we have been successful in the introduction of expression cassettes up to ~3.1 kb in length (P_{rrn}-*glpB-hph-aadA*-T_{psbA}). In the future, it might be desirable to possess the capability of introducing up to 10 kb or more of foreign DNA (perhaps an operon comprising a complex, multi-step biosynthetic pathway), into the plastid chromosome. It seems quite predictable that as the size of the foreign DNA for integration increases, the efficiency of plastid transformation will concomitantly decline. However, Example 9 provides a method for effectively doubling the size limit (at this time) of foreign DNA that can be integrated into the plastid chromosome in a single transformation event.

The results described here also suggest that multiple homologous recombination events can be detected between plasmid and chromosomal templates during a single transformation event. This observation further suggests that co-bombarded plasmids that are targeted for integration at different loci on the plastid chromosome have a strong likelihood of being recovered in a single transformation event. This capability would increase the rate at which multiple genes could be manipulated during a single round of transformation. Collectively, these observations indicate that co-transformation of two (or more) different DNA templates will greatly enhance our ability to genetically manipulate the plastid genome.

Example 10

Recovery of Phosphinothricin-Resistant Tobacco NT1 Cell Plastid Transformants Expressing the bar Gene

The successful utilization of the *hph*, *glpA*, and *glpB* genes to confer glyphosate resistance in plastid transformants of a number of land plant species prompted the question as to whether there were other available genes that might also confer herbicide resistance when expressed in the plastid. The non-selective herbicide, LIBERTY® (also known as BASTA®) contains the active ingredient, phosphinothricin (PPT; also known as glufosinate, which terms are used interchangeably herein). PPT acts by inhibiting the action of glutamine synthetase (GS), a nuclear-encoded amino acid biosynthetic enzyme (for glutamine) whose activity is localized primarily in the plastid. Plant cells exposed to PPT, a glutamate analogue, become impaired in their nitrogen metabolism and not only accumulate high levels of ammonia, but also become starved for glutamine. Transformed plant cells expressing the *Streptomyces bar* gene inactivate PPT by the process of acetylation, thus preventing both the accumulation of ammonia and depletion

of glutamine. We envisioned that plastid expression of the *bar* gene would acetylate the PPT as it entered the organelle, thereby providing resistance to the plant cell.

Unanticipated cloning problems presumably associated with *bar* over-expression in *E. coli* required the construction of two complementary vectors. Briefly, a *bar*-containing plasmid was assembled that alone could not express *bar* acetyltransferase activity well, if at all, in *E. coli* or plastids. However, if co-bombarded with an accompanying plasmid, and taking advantage of the active homologous recombination system in this organelle, recombination between the two plasmids could lead to restoration of full and complete *bar* expression. Co-bombardment of the two plasmids into tobacco NT1 cells yielded glyphosate-resistant plastid transformants that were then analyzed for their ability to grow on PPT-containing medium.

Materials and Methods for Example 10

Plasmid construction. For the construction of plasmid pSCO56, plasmid pSCO35, which contains the P_{rrn}-*hph*-T_{rbcL} expression cassette inserted into the *Hinc* II site at the end of ORF70B in plasmid pSAN307, was partially digested with restriction endonuclease *Sca* I. *Sca* I cleaves near the 3′ end of the *hph* coding region (as well as once within the *bla* gene of the vector backbone). The partially-digested plasmid DNA was digested again with *Eco* RV, which cleaves once in the vicinity of the *rps12* gene near the end of the flanking chloroplast DNA. A linear DNA fragment that now lacks a portion of the *hph* coding sequence, the T_{rbcL} element for plastid transcript maturation and stability, and virtually the entire 3′ flanking chloroplast region was isolated by gel purification. This DNA fragment was then re-ligated to form pSCO56.

For the construction of plasmid pSCO57, plasmid pSCO34, which contains the P_{rrn}-T_{rbcL} expression cassette inserted into the *Hinc* II site at the end of ORF70B in

plasmid pSAN307, was digested with *Hpa* I and *Bam* HI to liberate a 1.2 kb fragment containing the T_{rbcL} element and the entire 3′-flanking chloroplast DNA region. At the same time, plasmid pSCO26, which contains the *bar* coding region (with its own plastid-like RBS element) in Bluescript, was digested with *Sma* I and *Bam* HI, both of which cleave immediately downstream of the *bar* gene. The 1.2 kb *Hpa* I - *Bam* HI T_{rbcL}-chloroplast DNA fragment from pSCO34 was then inserted between the *Sma* I and *Bam* HI sites of pSCO26 so that the *bar* gene was now flanked at its 3′ end by the T_{rbcL} element and associated 3′-flanking chloroplast sequences. The resulting plasmid was then linearized with *Xho* I, which cleaves within the MCS region just prior to the 5′ end of the *bar* gene. The fragment ends were then treated with T4 DNA polymerase to create blunt ends for the next cloning step. Plasmid pSCO32, which contains the *hph* coding region (also with its own plastid-like RBS element), was digested with *Xho* I and *Sma* I to liberate a 1.1 kb *hph*-containing fragment. The *hph* fragment was then treated with T4 DNA polymerase to fill in the *Xho* I ends. The blunt-ended *hph* fragment was then inserted into the modified *Xho* I site of the plasmid described immediately above. The resulting plasmid, pSCO57, contains in order, promoterless *hph* and *bar* genes arranged in a dicistron, the T_{rbcL} element and finally, ~0.9 kb of petunia chloroplast DNA.

Plant cell transformation. Plant cell transformation was carried out as described in Example 2.

Results and Discussion

As demonstrated herein, transplastomic tobacco plants expressing *hph* or *glpB-hph* manifested commercially-significant levels of resistance to the herbicide, ROUNDUP®. To create additional transplastomic herbicide-resistant plants, we considered other herbicides that act upon the plastid and any associated herbicide

resistance genes that might be more efficacious when expressed in plastids rather than the nucleus. Phosphinothricin (PPT), the active ingredient in the non-selective herbicide, LIBERTY®, acts by inhibiting the action of glutamine synthetase (GS), an amino acid biosynthetic enzyme (for glutamine) and the main enzyme responsible for nitrogen metabolism in the plant cell. Glutamine synthetase carries out the enzymatic conversion of glutamate (or glutamic acid) to glutamine in an ATP-dependent reaction. Plant cells exposed to PPT, a glutamate analogue, become impaired in their nitrogen metabolism and not only accumulate high levels of ammonia but also become starved for glutamine. The bacterial *bar* and *pat* genes, isolated from individual *Streptomyces* species, confer resistance to phosphinothricin (PPT). Transformed plant cells expressing the *bar* or *pat* gene inactivate PPT by the process of acetylation (the proteins possess acetyltransferase activity), thus preventing both the accumulation of ammonia and depletion of glutamine.

In the plant systems thus far studied (*e.g.*, *Arabidopsis thaliana*), glutamine synthetase activity is conferred by both plastid- and cytosolic-localized enzymes encoded by nuclear genes. Lam *et al.*, *The Plant Cell* 7, 887 (1995). The plastid-localized GS2 enzyme, encoded by the *GLN2* gene, is expressed strongly throughout photosynthetic tissues and is thought to be largely responsible for the roles of glutamine biosynthesis and nitrogen assimilation within the plant. The function of the cytosolic GS1, expressed most strongly in roots from the GLN1 gene (or members of the GLN1 gene family), is less certain. Since the chloroplast-localized GS2 protein is the likely primary target of PPT action, high-level expression of the *bar* gene in the plastid should be sufficient to confer significant levels of PPT resistance to the plant cell.

A question remained as to whether plastid-localized *bar* expression would adequately protect the cytosolic version of glutamine synthetase. However, it should be recalled that PPT, unlike glyphosate, is not efficiently translocated throughout the plant.

Thus, after herbicide application, PPT levels should be relatively modest in the root, the tissue that shows the highest level of GLN1 expression in the plant. Therefore, the requirement for protection of cytosolic GS1 is likely obviated.

To assess whether a plastid-expressed *bar* (or *pat*) gene could confer PPT resistance to plants sprayed with the herbicide LIBERTY®, we modified the *bar* gene to be placed under control of the petunia plastid *rrn* promoter. The *bar* gene, supplied with its own RBS element based upon the *rbcL* gene (the same RBS element as employed for *hph*, *glpA* and *glpB* genes expressed in the plastid), was ligated between the petunia plastid *rrn* promoter and the *aadA* gene such that a dicistronic *bar-aadA* transcript would be predicted to be synthesized in the plastid. Surprisingly, no desired recombinants could be recovered in *E. coli*. Additional experiments confirmed that both the vector and insert DNA's had the proper restriction ends and could be successfully utilized in other cloning procedures. The repeated inability to recover the desired P_{rrn}-*bar-aadA*-T_{psbA} transgene was similar to the cloning problems experienced with *glpA* and led us to consider the possibility that overexpression of *bar* in *E. coli* might somehow be lethal. Therefore, we devised a cloning strategy similar to that employed for *glpA* to overcome this obstacle.

The general strategy adopted was to construct two complementary plastid expression vectors that, when recombined within the plastid, would permit *bar* expression. Some uncertainty existed as to whether the *bar* gene could act both as a herbicide resistance gene and also as a selectable marker gene for plastid transformation (as there was serious concern that rapid, PPT-induced ammonia accumulation might be toxic to the plant cell before sufficient *bar* gene amplification in the plastids had occurred). In the event that the *bar* gene did not permit direct recovery of PPT-resistant plastid transformants, the *hph* gene was included to permit recovery of glyphosate-

resistant, *bar*-expressing plastid transformants that could later be assessed for their PPT resistance phenotype.

One plasmid, designated pSCO56, contained a plastid gene expression cassette with a truncated version of the *hph* gene (designated **hph**) under control of the petunia plastid *rrn* promoter. Besides missing a portion of the carboxyl-terminus of the HPH protein, this cassette lacked a plastid 3′ element for transcript maturation and stability and was flanked on just one side (rather than the usual two sides) with chloroplast DNA necessary for facilitating integration into the plastid chromosome. It was anticipated that this plasmid alone would not permit recovery of glyphosate-resistant plastid transformants since both DNA integration (via double homologous recombination events) and HPH enzymatic activity would be severely impaired. A second plasmid, designated pSCO57, contained in order, promoterless *hph* and *bar* genes, the T_{rbcL} element for plastid transcript maturation and stability, and flanking chloroplast DNA sequences for facilitating DNA integration. Although the *hph* and *bar* genes each possessed their own respective plastid-like RBS elements, no plastid promoter was linked to the genes, thereby avoiding the putative lethality problem associated with *bar* overexpression in *E. coli*. Once again, it was anticipated that plastid transformation with plasmid pSCO57 alone would not confer glyphosate resistance for the same reasons provided above for plasmid pSCO56.

After co-bombardment of plasmids pSCO56 and pSCO57 into the plastid, however, homologous recombination between their respective *hph* genes (the only shared regions of homology within the cassette) should yield a fully functional P_{rrn}-*hph* -*bar*-T_{rbcL} cassette capable of integrating into the plastid chromosome. Recombination within the *hph* sequences of pSCO56 and pSCO57 should preferentially occur extra-chromosomally (plasmid-to-plasmid and not plasmid-to-chromosome) since neither of these plasmids

should integrate with much efficiency into the plastid chromosome due to insufficient regions of homology.

This differs significantly from the very similar approach used for expressing *glpA* within the plastid. Plasmid pSCO24, harboring the mutant *glpA* gene, was capable of integrating into the plastid chromosome. Therefore, double homologous recombination events between plasmid pSCO18 (carrying the wild-type *glpA* gene) and sequences on pSCO24 could potentially occur as either plasmid-to-plasmid or plasmid-to-chromosome events.

Plasmids pSCO56 and pSCO57 were co-precipitated onto tungsten for bombardment into tobacco NT1 cells, followed by selection on medium containing 2 mM glyphosate. Cells resistant to glyphosate will be observed to manifest both successful transformation and resultant glyphosate and glufosinate resistance.

We claim:

1. A method of producing an herbicide-resistant plant cell, the method comprising stably transforming a plastid or proplastid genome of the plant cell with a nucleic acid comprising a first herbicide-resistance-conferring selectable marker gene, wherein the first selectable marker gene encodes a protein that inactivates the herbicide, thereby eliminating the toxicity of the herbicide to the plant cell, and which gene is expressed at levels that result in the plant cell surviving contact with the minimal amount of the herbicide that would kill an untransformed plant cell of the same species.

2. The method according to claim 1, wherein the herbicide is glyphosate.

3. The method according to claim 1, wherein the herbicide is glufosinate.

4. The method according to claim 2, wherein the first glyphosate resistance selectable marker gene is the *hph* gene.

5. The method according to claim 2, wherein the first glyphosate resistance selectable marker gene is the *glpA* gene.

6. The method according to claim 3, wherein the first glufosinate resistance selectable marker gene is the *bar* gene.

7. The method according to claim 3, wherein the first glufosinate resistance selectable marker gene is the *pat* gene.

8. The method according to claim 1, wherein the nucleic acid comprises a plurality of genes.

9. The method according to claim 1, wherein the nucleic acid further comprises a second gene.

10. The method according to claim 9, wherein the first herbicide resistance-conferring gene is a glyphosate resistance-conferring selectable marker gene and the second gene is a different herbicide resistance-conferring selectable marker gene.

11. The method according to claim 10, wherein the first selectable marker gene is the *hph* or *glpA* gene.

12. The method according to claim 11, wherein the second gene encodes a glyphosate resistant EPSPS enzyme.

13. The method according to claim 10, wherein the second gene is either the *bar* gene or the *pat* gene.

14. The method according to claim 9, wherein the second selectable marker gene is other than a herbicide resistance-conferring gene.

15. The method according to claim 14, wherein the first selectable marker gene is a glyphosate resistance-conferring gene and the second gene encodes a protein that enhances the glyphosate resistance of a plant cell whose plastids are transformed with and express both genes.

16. The method according to claim 15, wherein the first selectable marker gene is *hph* or *glpA* and the second gene is *glpB*.

17. The method according to claim 9, wherein the nucleic acid further comprises a third gene.

18. The method according to claim 17, wherein the first gene is a glyphosate resistance conferring gene.

19. The method according to claim 18, wherein the glyphosate resistance conferring gene is *hph* or *glpA*.

20. The method according to claim 19, wherein the glyphosate resistance conferring gene is *hph* or *glpA*, the second gene is *glpB*, and the third gene is the *bar* or *pat* gene.

21. The method according to claim 1, wherein the plant cell is a non-photosynthetic cell.

22. The method according to claim 4, wherein the plant cell is a non-photosynthetic cell.

23. The method according to claim 5, wherein the plant cell is a non-photosynthetic cell.

24. The method according to claim 9, wherein the plant cell is a non-photosynthetic cell.

25. The method according to claim 11, wherein the plant cell is a non-photosynthetic cell.

26. The method according to claim 16, wherein the plant cell is a non-photosynthetic cell.

27. The method according to claim 17, wherein the plant cell is a non-photosynthetic cell.

28. The method according to claim 19, wherein the plant cell is a non-photosynthetic cell.

29. The method according to claim 1, wherein the plant cell is a monocot plant cell.

30. The method according to claim 4, wherein the plant cell is a monocot plant cell.

31. The method according to claim 5, wherein the plant cell is a monocot plant cell.

32. The method according to claim 9, wherein the plant cell is a monocot plant cell.

33. The method according to claim 11, wherein the plant cell is a monocot plant cell.

34. The method according to claim 16, wherein the plant cell is a monocot plant cell.

35. The method according to claim 17, wherein the plant cell is a monocot plant cell.

36. The method according to claim 19, wherein the plant cell is a monocot plant cell.

37. The method according to any one of claims 21 to 28, wherein the plant cell is a monocot plant cell.

38. The method according to any one of claims 29 to 36, wherein the monocot is selected from the group consisting of wheat, rice, maize, and turfgrass.

39. The method according to claim 37, wherein the monocot is selected from the group consisting of wheat, rice, maize, and turfgrass.

40. The method according to claim 1, wherein said transforming comprises introduction of a first vector and a second vector into the plastid, wherein

a) the first vector comprises an herbicide resistance-conferring selectable marker gene whose expression product is capable of inactivating an herbicide, but which vector does not comprise one or a plurality of nucleic acid sequences required for recombination into the plastid or proplastid genome, required for expression of the selectable marker gene, or both,

b) the second vector comprises the nucleic acid sequence or sequences not present in the first vector that are required for recombination into the plastid or proplastid genome, required for expression of the selectable marker gene, or both,

and wherein the first vector, the second vector, and the plastid genome together are capable of recombining through a series of recombination events to produce a plastid genome transformed with the herbicide resistance-conferring selectable marker gene.

41. The method according to claim 40, wherein the herbicide resistance-conferring selectable marker gene is the *glpA* gene.

42. The method according to claim 40, wherein the herbicide resistance-conferring selectable marker gene is the *bar* gene.

43. The method according to claim 41 or 42, wherein the plant cell is a non-photosynthetic cell.

44. The method according to claim 43, wherein the plant cell is a monocot plant cell.

45. The method according to claim 44, wherein the monocot is selected from the group consisting of wheat, rice, maize, and turfgrass.

46. A multicellular plant tissue resistant to an herbicide, the plant tissue comprising a plurality of cells having plastids, proplastids, or both whose genome comprise a first herbicide-resistance-conferring selectable marker gene, wherein the first selectable marker gene encodes a protein that inactivates the herbicide, thereby eliminating the toxicity of the herbicide to the cells, and which gene is expressed at levels that result in the plant tissue surviving contact with the minimal amount of the herbicide that would kill an untransformed plant tissue of the same species.

47. A method of transforming a plastid or proplastid of a plant cell, the method comprising simultaneously introducing two plasmids into the plastid or proplastid.

48. The method according to claim 47, wherein one of the plasmids comprises an herbicide resistance conferring selectable marker gene.

49. The method according to claim 48, wherein the herbicide resistance conferring selectable marker gene is the *hph* or *glpA* gene.

50. The multicellular plant tissue according to claim 46, wherein the herbicide is glyphosate.

51. The multicellular plant tissue according to claim 46, wherein the herbicide is glufosinate.

52. The multicellular plant tissue according to claim 50, wherein the first glyphosate resistance selectable marker gene is the *hph* gene.

53. The multicellular plant tissue according to claim 50, wherein the first glyphosate resistance selectable marker gene is the *glpA* gene.

54. The multicellular plant tissue according to claim 51, wherein the first glufosinate resistance selectable marker gene is the *bar* gene.

55. The multicellular plant tissue according to claim 51, wherein the first glufosinate resistance selectable marker gene is the *pat* gene.

56. The multicellular plant tissue according to claim 46, wherein the nucleic acid comprises a plurality of genes.

57. The multicellular plant tissue according to claim 56, wherein the nucleic acid comprises a second gene.

58. The multicellular plant tissue according to claim 57, wherein the first herbicide resistance-conferring gene is a glyphosate resistance-conferring selectable marker gene and the second gene is a different herbicide resistance-conferring selectable marker gene.

59. The multicellular plant tissue according to claim 58, wherein the first selectable marker gene is the *hph* or *glpA* gene.

60. The multicellular plant tissue according to claim 59, wherein the second gene encodes a mutant EPSPS enzyme.

61. The multicellular plant tissue according to claim 58, wherein the second gene is either the *bar* gene or the *pat* gene.

62. The multicellular plant tissue according to claim 57, wherein the second selectable marker gene is other than a herbicide-resistance-conferring gene.

63. The multicellular plant tissue according to claim 62, wherein the first selectable marker gene is a glyphosate resistance-conferring gene and the second gene encodes a protein that enhances the glyphosate resistance of a plant cell whose plastids are transformed with and express both genes.

64. The multicellular plant tissue according to claim 63, wherein the first selectable marker gene is *hph* or *glpA* and the second gene is *glpB*.

65. The multicellular plant tissue according to claim 57, wherein the nucleic acid further comprises a third gene.

66. The multicellular plant tissue according to claim 65, wherein the first gene is a glyphosate resistance conferring gene.

67. The multicellular plant tissue according to claim 66, wherein the glyphosate resistance conferring gene is *hph* or *glpA*.

68. The multicellular plant tissue according to claim 67, wherein the glyphosate resistance conferring gene is *hph* or *glpA*, the second gene is *glpB*, and the third gene is the *bar* or *pat* gene.

69. The multicellular plant tissue according to claim 46, wherein the plant cell is a non-photosynthetic cell.

70. The multicellular plant tissue according to claim 52, wherein the plant cell is a non-photosynthetic cell.

71. The multicellular plant tissue according to claim 53, wherein the plant cell is a non-photosynthetic cell.

72. The multicellular plant tissue according to claim 57, wherein the plant cell is a non-photosynthetic cell.

73. The multicellular plant tissue according to claim 59, wherein the plant cell is a non-photosynthetic cell.

74. The multicellular plant tissue according to claim 64, wherein the plant cell is a non-photosynthetic cell.

75. The multicellular plant tissue according to claim 65, wherein the plant cell is a non-photosynthetic cell.

76. The multicellular plant tissue according to claim 67, wherein the plant cell is a non-photosynthetic cell.

77. The multicellular plant tissue according to claim 46, wherein the plant cell is a monocot plant cell.

78. The multicellular plant tissue according to claim 52, wherein the plant cell is a monocot plant cell.

79. The multicellular plant tissue according to claim 53, wherein the plant cell is a monocot plant cell.

80. The multicellular plant tissue according to claim 57, wherein the plant cell is a monocot plant cell.

81. The multicellular plant tissue according to claim 59, wherein the plant cell is a monocot plant cell.

82. The multicellular plant tissue according to claim 64, wherein the plant cell is a monocot plant cell.

83. The multicellular plant tissue according to claim 65, wherein the plant cell is a monocot plant cell.

84. The multicellular plant tissue according to claim 67, wherein the plant cell is a monocot plant cell.

85. The multicellular plant tissue according to any one of claims 69 to 76, wherein the plant cell is a monocot plant cell.

86. The multicellular plant tissue according to any one of claims 77 to 84 , wherein the monocot is selected from the group consisting of wheat, rice, maize, and turfgrass.

87. The multicellular plant tissue according to claim 85, wherein the monocot is selected from the group consisting of wheat, rice, maize, and turfgrass.

88. A nucleic acid comprising:

a) an herbicide resistance-conferring selectable marker gene whose expression product inactivates an herbicide, and

b) flanking sequences homologous to regions of the plastid genome, which flanking sequences enable the nucleic acid to integrate by recombination into the plastid genome.

89. The nucleic acid according to claim 88, wherein the herbicide resistance-conferring selectable marker gene is a glyphosate resistance-conferring gene.

90. The nucleic acid according to claim 89, wherein the glyphosate resistance-conferring gene is the *hph* or *glpA* gene.

91. The nucleic acid according to claim 90, wherein the plastid promoter is the *rrn* promoter.

92. The nucleic acid according to claim 90, wherein the flanking regions are homologous to the plastid genome inverted repeat region.

93. The nucleic acid according to claim 88, wherein the nucleic acid comprises a plurality of genes.

94. The nucleic acid according to claim 93, wherein the nucleic acid comprises a second gene.

95. The nucleic acid according to claim 94, wherein the first herbicide resistance-conferring gene is a glyphosate resistance-conferring selectable marker gene and the second gene is a different herbicide resistance-conferring selectable marker gene.

96. The nucleic acid according to claim 95, wherein the first selectable marker gene is the *hph* or *glpA* gene.

97. The nucleic acid according to claim 96, wherein the second gene encodes a mutant EPSPS enzyme.

98. The nucleic acid according to claim 95, wherein the second gene is either the *bar* gene or the *pat* gene.

99. The nucleic acid according to claim 94, wherein the second selectable marker gene is other than a herbicide-resistance-conferring gene.

100. The nucleic acid according to claim 99, wherein the first selectable marker gene is a glyphosate resistance-conferring gene and the second gene encodes a protein that enhances the glyphosate resistance of a plant cell whose plastids are transformed with and express both genes.

101. The nucleic acid according to claim 100, wherein the first selectable marker gene is *hph* or *glpA* and the second gene is *glpB*.

102. The nucleic acid according to claim 94, wherein the nucleic acid further comprises a third gene.

103. The nucleic acid according to claim 102 wherein the first gene is a glyphosate resistance conferring gene.

104. The nucleic acid according to claim 103, wherein the glyphosate resistance conferring gene is *hph* or *glpA*.

105. The nucleic acid according to claim 104, wherein the glyphosate resistance conferring gene is *hph* or *glpA*, the second gene is *glpB*, and the third gene is the *bar* or *pat* gene.

106. The nucleic acid according to and one of claims 94 to 105, wherein the plastid promoter is the *rrn* promoter.

107. The nucleic acid according to and one of claims 94 to 106, wherein the flanking regions are homologous to the plastid genome inverted repeat region.

108. A composition of two vectors, wherein

a) the first vector comprises an herbicide resistance-conferring selectable marker gene whose expression product is capable of inactivating an herbicide, but which vector does not comprise one or a plurality of nucleic acid sequences required for recombination into the plastid or proplastid genome, required for expression of the selectable marker gene, or both, and

b) the second vector comprises the nucleic acid sequence or sequences not present in the first vector that are required for recombination into the plastid or proplastid genome, required for expression of the selectable marker gene, or both,

such that when the composition is introduced into the plastid, the first and second vector, together with the plastid genome can recombine to yield a transformed plastid genome

capable of expressing the herbicide resistance-conferring selectable marker gene at levels sufficient to confer herbicide resistance to amount of the herbicide that would kill an untransformed cell of the same species.

109. The composition according to claim 108, wherein the herbicide resistance-conferring selectable marker gene is the *glpA* gene.

110. The composition according to claim 108, wherein the herbicide resistance-conferring selectable marker gene is the *bar* gene.

111. The composition according to claim 109 or 110, wherein the plant cell is a non-photosynthetic cell.

112. The composition according to claim 111, wherein the plant cell is a monocot plant cell.

113. The composition according to claim 112, wherein the monocot is selected from the group consisting of wheat, rice, maize, and turfgrass.

114. A method of producing transformed non-photosynthetic plant cells, the method comprising transforming a non-photosynthetic plant cell with a nucleic acid comprising the *aadA* gene, wherein the *aadA* gene is expressed at levels that result in the plant cell surviving contact with the minimal amount of spectinomycin that would kill an untransformed plant cell of the same species.

115. The method according to claim 114, wherein the nucleic acid comprises a plurality of genes.

116. The method according to claim 114, wherein the nucleic acid further comprises a second gene.

117. The method according to claim 115, wherein the second gene is a glyphosate resistance-conferring gene.

118. The method according to claim 117, wherein the second gene is the *hph* or *glpA* gene.

119. The method according to any one of claims 107 to 118, wherein the plant cell is a monocot plant cell.

120. The method according to any one of claims 119, wherein the monocot is selected from the group consisting of wheat, rice, maize, and turfgrass.

121. A multicellular plant tissue comprising a plurality of non-photosynthetic cells transformed with a nucleic acid comprising the *aadA* gene, wherein the *aadA* gene is expressed at levels that result in the plant cell surviving contact with the minimal amount of spectinomycin that would kill an untransformed plant cell of the same species.

122. The multicellular plant tissue according to claim 121, wherein the nucleic acid comprises a plurality of genes.

123. The multicellular plant tissue according to claim 121, wherein the nucleic acid further comprises a second gene.

124. The multicellular plant tissue according to claim 122, wherein the second gene is a glyphosate resistance-conferring gene.

125. The multicellular plant tissue according to claim 124, wherein the second gene is the *hph* or *glpA* gene.

126. The method according to any one of claims 121 to 125, wherein the plant cell is a monocot plant cell.

127. The method according to any one of claims 126, wherein the monocot is selected from the group consisting of wheat, rice, maize, and turfgrass.

FIG. 1

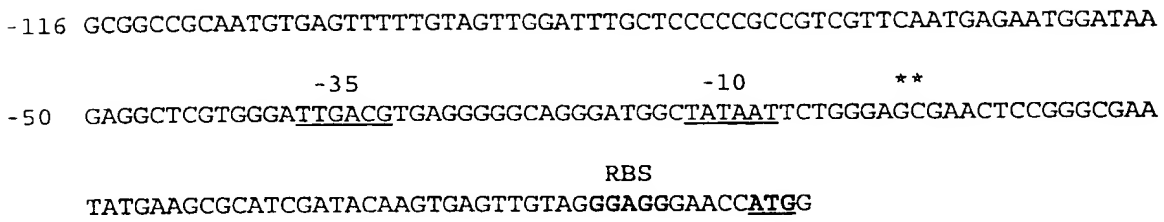

-116 GCGGCCGCAATGTGAGTTTTTGTAGTTGGATTTGCTCCCCCGCCGTCGTTCAATGAGAATGGATAA
-35
-10
**
-50 GAGGCTCGTGGGATTGACGTGAGGGGGCAGGGATGGCTATAATTCTGGGAGCGAACTCCGGGCGAA
RBS
TATGAAGCGCATCGATACAAGTGAGTTGTAGGGAGGGAACCATGG

FIG. 2

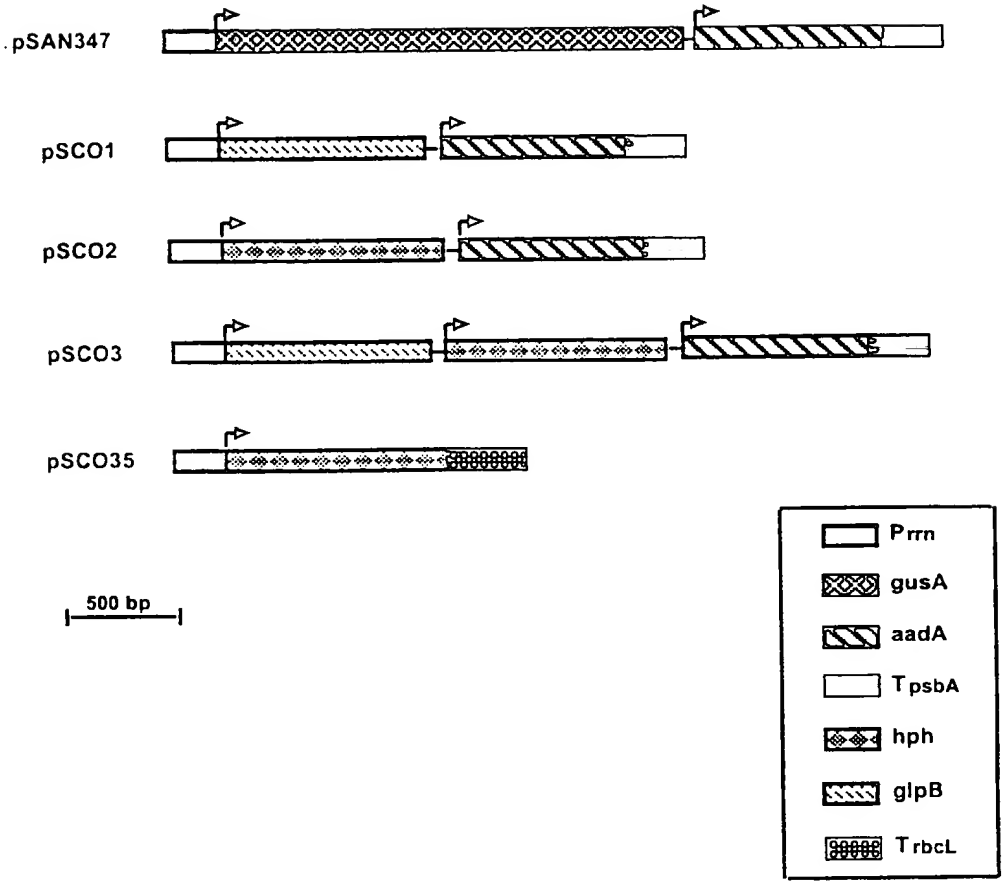
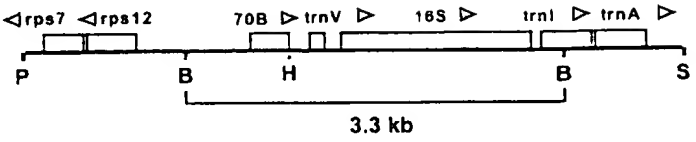
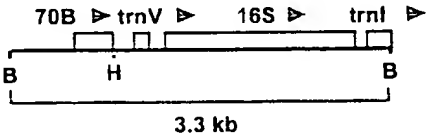

pSAN347
pSCO1
pSCO2
pSCO3
pSCO35
500 bp
Prrn
gusA
aadA
TpsbA
hph
glpB
TrbcL

FIG. 3

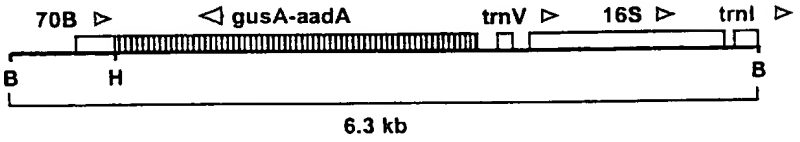
A pSAN347 plastid targeting DNA



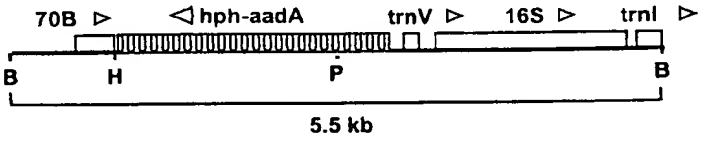
B pSCO1-pSCO3 plastid targeting DNA



C pSAN347 transgenic chromosome



D pSCO2 transgenic chromosome



E pSCO3 transgenic chromosome

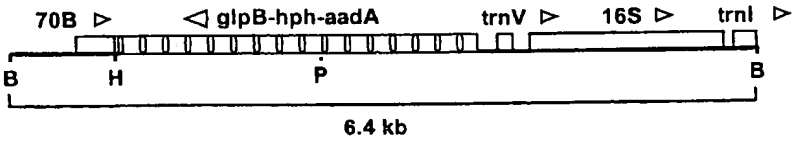


FIG. 4A

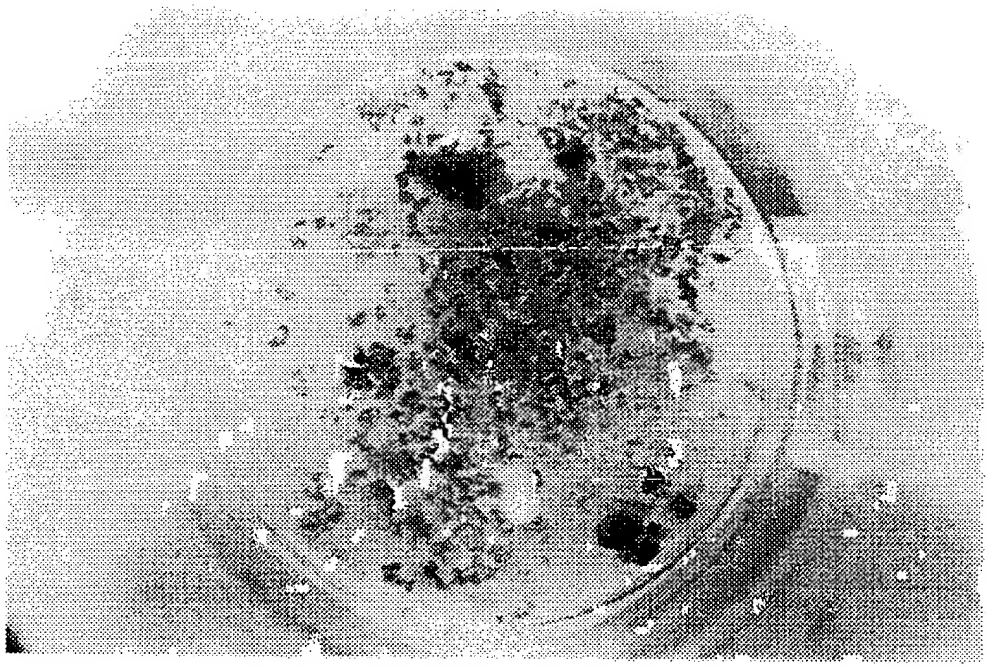


FIG. 4B

Sample	Reporter Activity[a]
Control	7
pSAN347	1.23×10^4
pBI426	0.44×10^4

[a] nmoles/mg protein/hr

FIG. 5

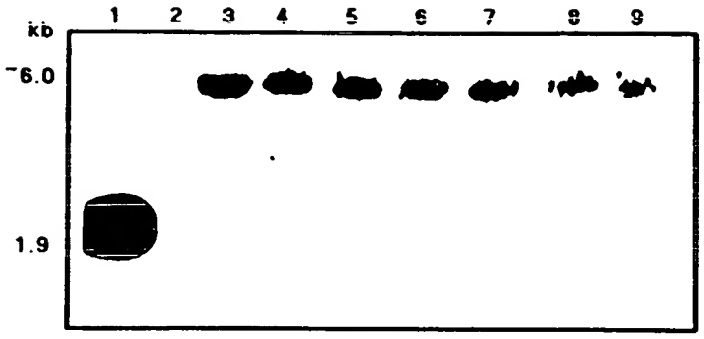


FIG. 6

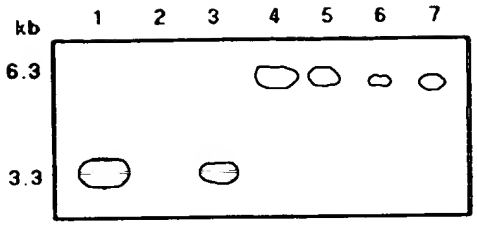


FIG. 7

Sample	Phosphotransferase activity[a] (x 10^{-3})
NT1 Control	0.62
pSCO2-1/NT1	4.48
pSCO2-2/NT1	17.84
pSCO2-3/NT1	10.50
pSCO3-1/NT1	8.19
pSCO3-2/NT1	8.95
pSCO3-3/NT1	19.31
NT-R[b] Control	0.60
pSCO2-1/NT-R	14.31
pSCO3-1/NT-R	3.04
pSCO3-2/NT-R	6.53
pSCO3-3/NT-R	4.86

[a] cpm/mg protein
[b] regenerable, photosynthetically-active callus

FIG. 8

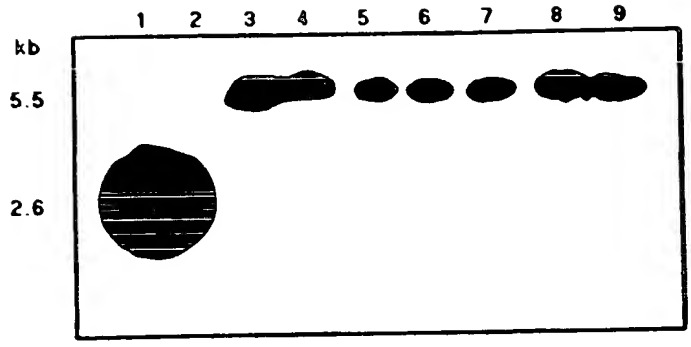


FIG. 9

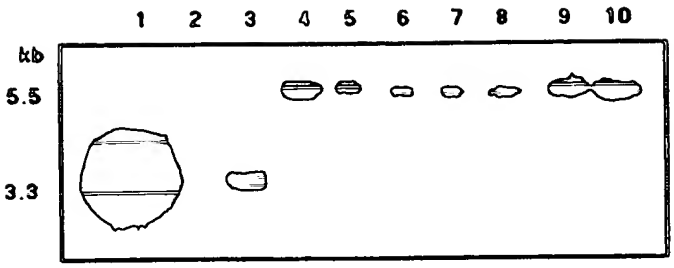


FIG. 10

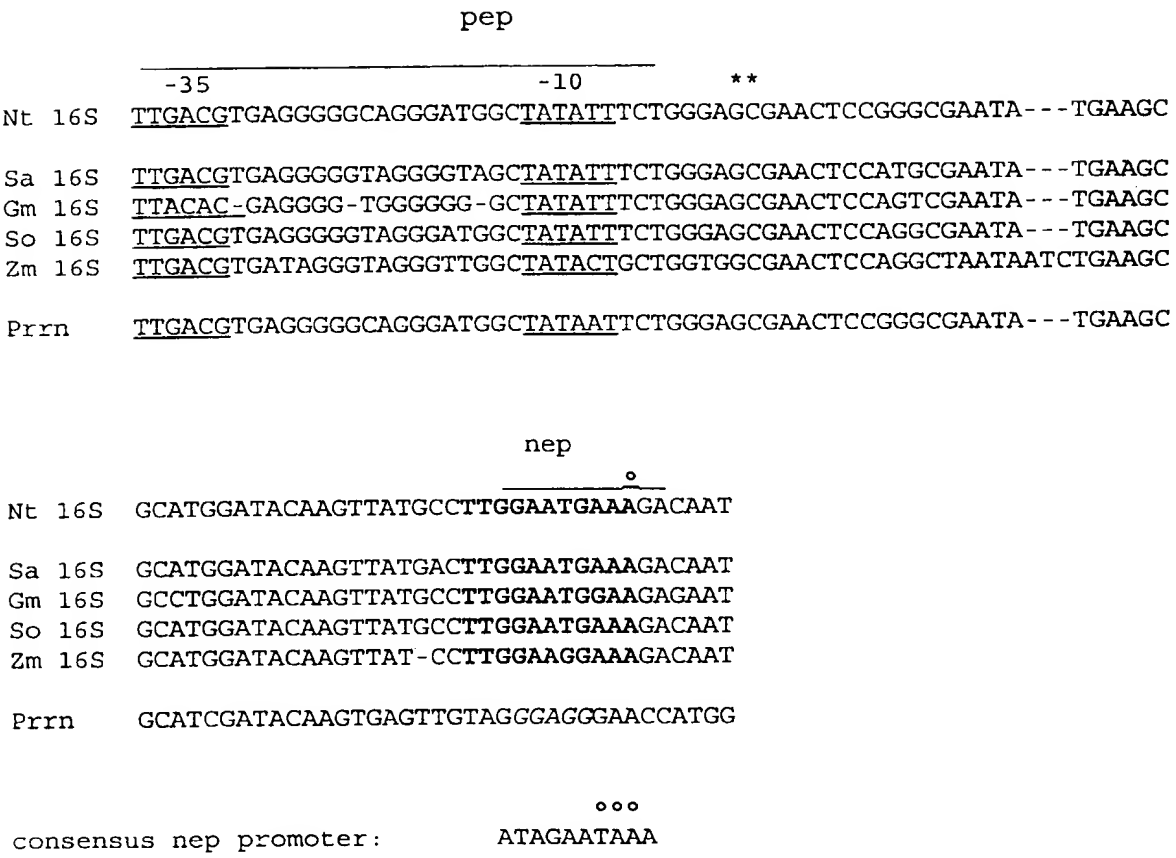

pep
-35
-10
**
Nt 16S TTGACGTGAGGGGGCAGGGATGGCTATATTTCTGGGAGCGAACTCCGGGCGAATA---TGAAGC
Sa 16S TTGACGTGAGGGGGTAGGGGTAGCTATATTTCTGGGAGCGAACTCCATGCGAATA---TGAAGC
Gm 16S TTACAC-GAGGGG-TGGGGGG-GCTATATTTCTGGGAGCGAACTCCAGTCGAATA---TGAAGC
So 16S TTGACGTGAGGGGGTAGGGATGGCTATATTTCTGGGAGCGAACTCCAGGCGAATA---TGAAGC
Zm 16S TTGACGTGATAGGGTAGGGTTGGCTATACTGCTGGTGGCGAACTCCAGGCTAATAATCTGAAGC
Prrn TTGACGTGAGGGGGCAGGGATGGCTATAATTCTGGGAGCGAACTCCGGGCGAATA---TGAAGC
nep
°
Nt 16S GCATGGATACAAGTTATGCCTTGGAATGAAAGACAAT
Sa 16S GCATGGATACAAGTTATGACTTGGAATGAAAGACAAT
Gm 16S GCCTGGATACAAGTTATGCCTTGGAATGGAAGAGAAT
So 16S GCATGGATACAAGTTATGCCTTGGAATGAAAGACAAT
Zm 16S GCATGGATACAAGTTAT-CCTTGGAAGGAAAGACAAT
Prrn GCATCGATACAAGTGAGTTGTAGGGAGGGAACCATGG
ooo
consensus nep promoter: ATAGAATAAA

FIG. 11

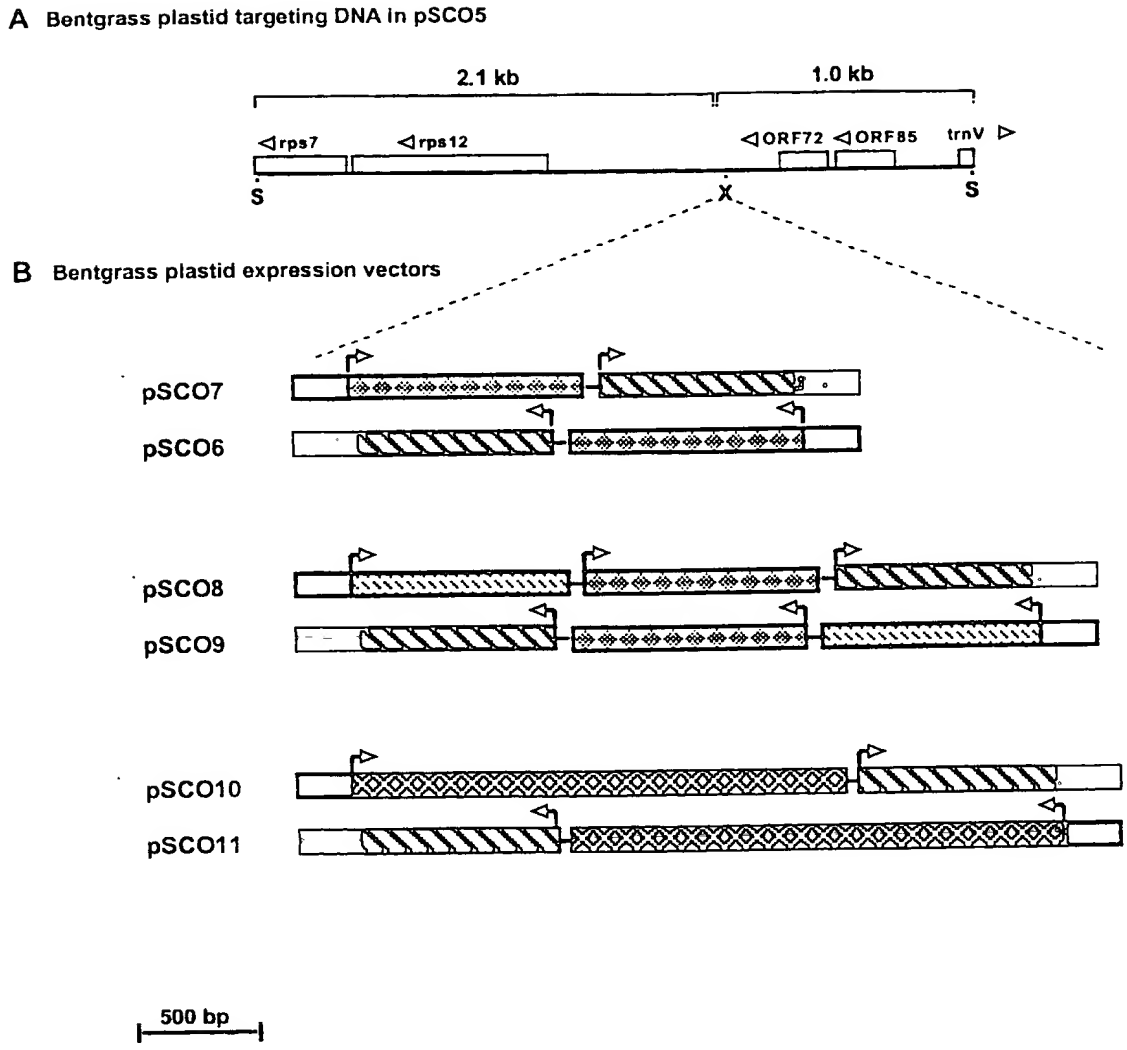

A Bentgrass plastid targeting DNA in pSCO5
2.1 kb
1.0 kb
rps7
rps12
ORF72
ORF85
trnV
S
X
S
B Bentgrass plastid expression vectors
pSCO7
pSCO6
pSCO8
pSCO9
pSCO10
pSCO11
500 bp

FIG. 12

Sample	Phosphotransferase activity[a] (x 10^{-3})
Tobacco Control (untrans.)	0.85
pSCO2-1	14.74
pSCO2-2	16.54
pSCO3-1	20.78
pSCO3-2	15.81
pSCO3-3	19.19

[a] cpm/mg protein

FIG. 13

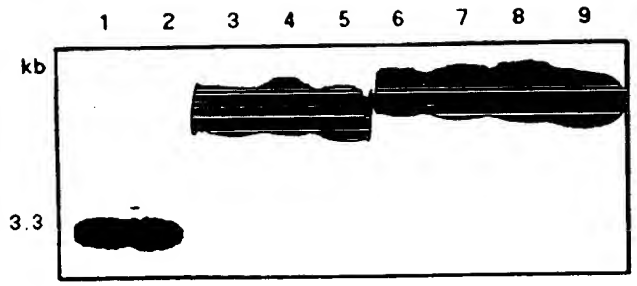


FIG. 14A

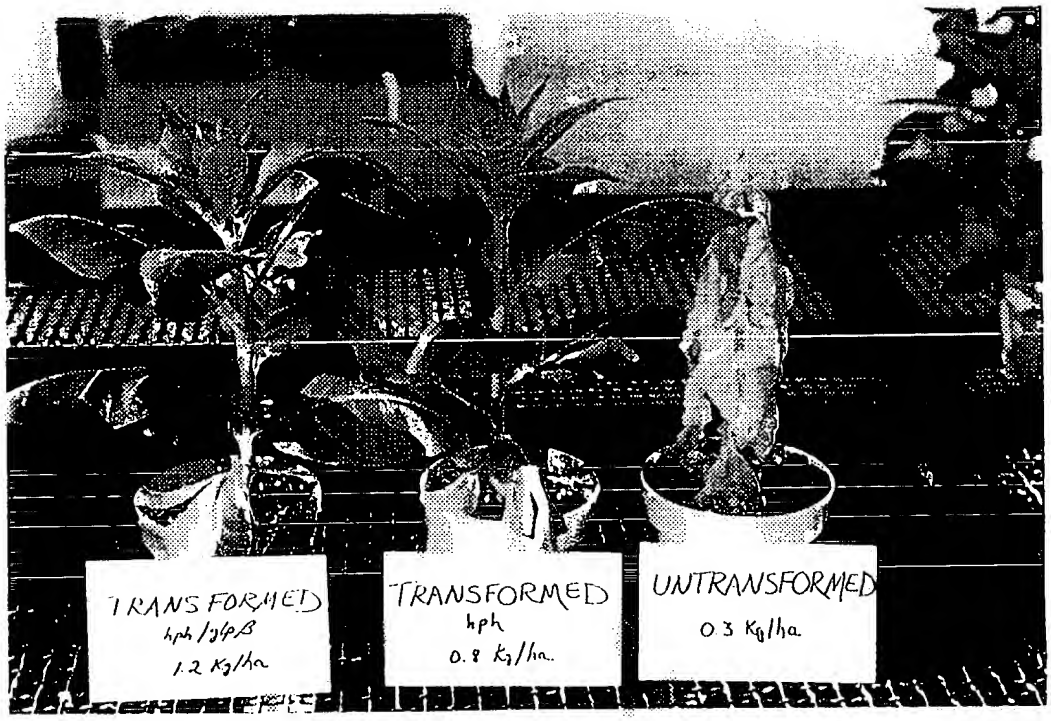


FIG. 14B

FIG. 15

Sample	Phosphotransferase activity[a] (x 10^{-3})
BMS Control (untrans.)	0.86
pSCO6-1	13.33
pSCO6-2	15.26
pSCO6-3	5.45
pSCO6-4	68.23
pSCO6-5	6.25

[a] cpm/mg protein

FIG. 16

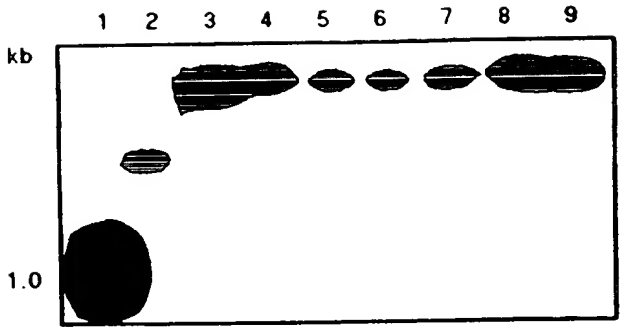


FIG. 17

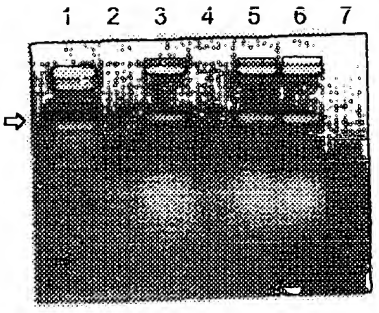


FIG. 18

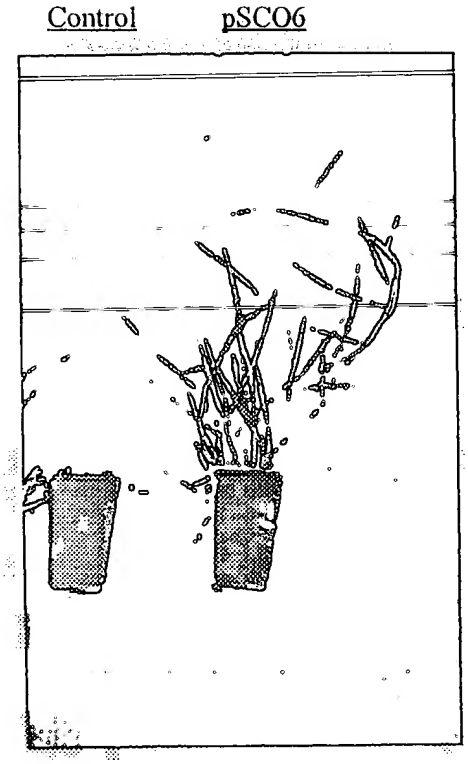


FIG. 19

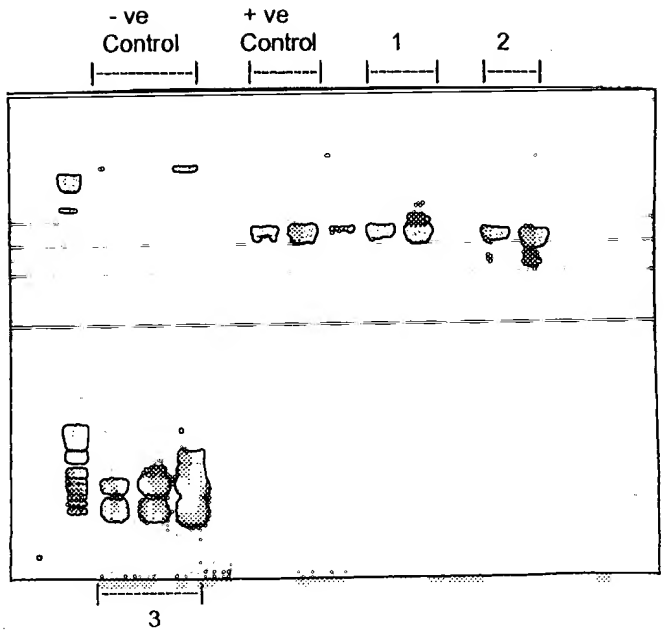


FIG. 20

Sample	Phosphotransferase activity[a] (x 10^{-3})
Avocado Control (untrans.)	0.85
pSCO2-1	2.54
pSCO2-2	4.74
pSCO2-3	4.11
pSCO2-4	31.14
pSCO2-5	24.82
Papaya Control (untrans.)	0.86
pSCO2-1	16.85
pSCO2-2	14.64

[a] cpm/mg protein

FIG. 21

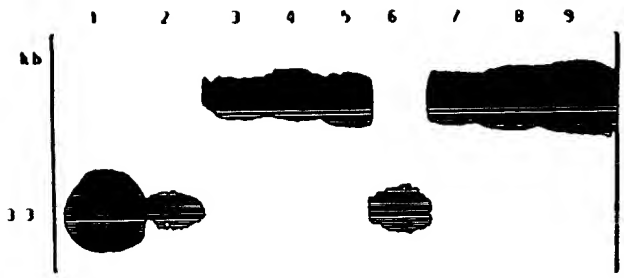


FIG. 22

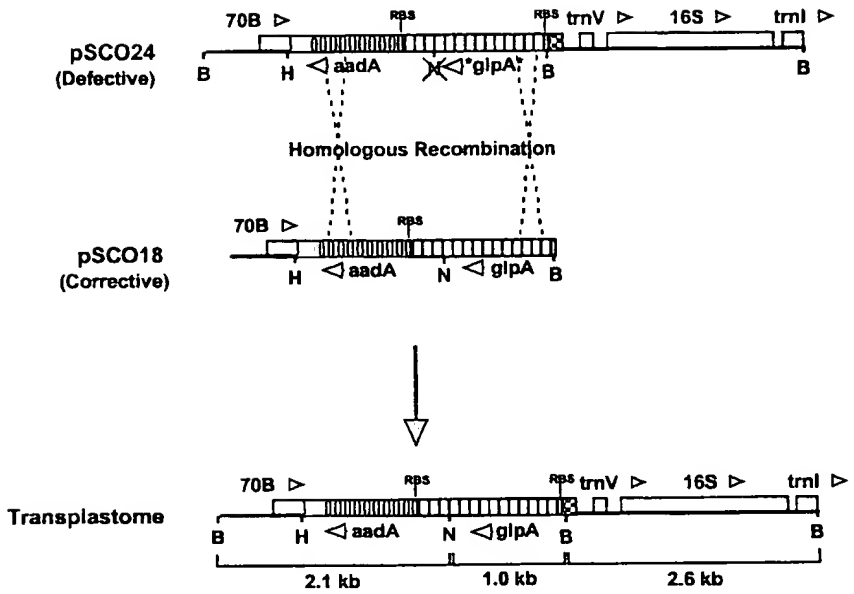

pSCO24
(Defective)
70B
RBS
RBS
trnV
16S
trnI
B
H
aadA
*glpA
B
B
Homologous Recombination
pSCO18
(Corrective)
70B
RBS
H
aadA
N
glpA
B
Transplastome
70B
RBS
RBS
trnV
16S
trnI
B
H
aadA
N
glpA
B
B
2.1 kb
1.0 kb
2.6 kb

FIG. 23

Sample	Phosphotransferase activity[a] (x 10^{-3})
NT1 Control (untrans.)	0.65
pSCO24/pSCO18-1	5.30
pSCO24/pSCO18-2	3.88
pSCO24/pSCO18-3	7.77
pSCO24/pSCO18-4	2.85

[a] cpm/mg protein

FIG. 24A

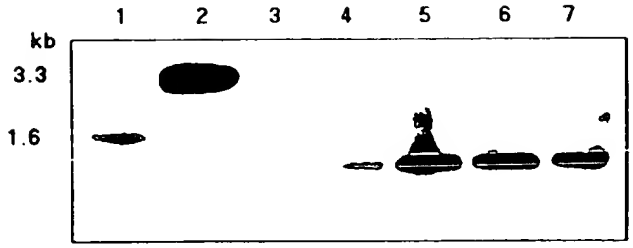


FIG. 24B

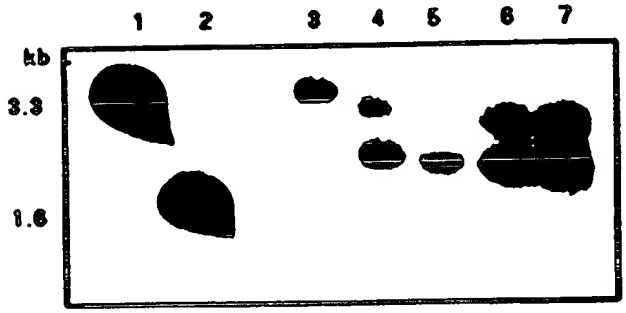
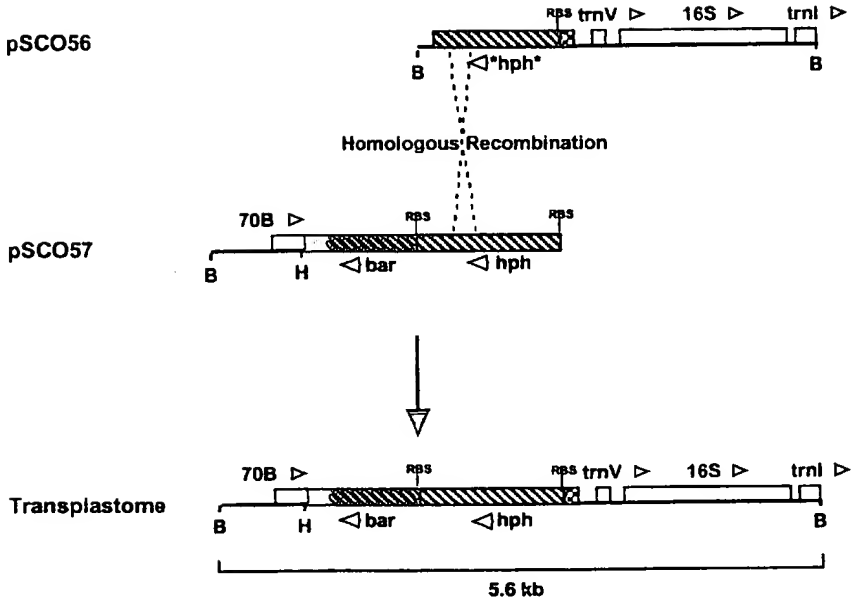


FIG. 25


pSCO56
RBS trnV 16S trnI
B
hph
B
Homologous Recombination
pSCO57
70B
RBS
RBS
B
H
bar
hph
Transplastome
70B
RBS
RBS trnV 16S trnI
B
H
bar
hph
B
5.6 kb

SEQUENCE LISTING

<110> Sandford Ph.D., John
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Centro De Investigacion y De Estudios Avanzados De

<120> IMPROVED PLASTID TRANSFORMATION OF HIGHER PLANTS AND PRODUCTION OF TRANSGENIC PLANTS WITH HERBICIDE RESISTANCE

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<160> 14

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transcription initiation site

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<223> Description of Artificial Sequence: derived from petunia chloroplast 16S rDNA promoter of the ribosomal RNA operon and the 16S rRNA transcription initiation site

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(54) Title: MULTIPLE GENE EXPRESSION FOR ENGINEERING NOVEL PATHWAYS AND HYPEREXPRESSION OF FOREIGN PROTEINS IN PLANTS

(57) Abstract: Introducing blocks of foreign genes in a single operon would avoid complications such as position effect and gene silencing inherent in putting one gene at a time into random locations in the nuclear genome. Cloning several genes into a single T-DNA does not avoid the compounded variable expression problem encountered in nuclear transgenic plants. This disclosure shows that a bacterial operon can be expressed in a single integration event as opposed to multiple events requiring several years to accomplish. Expression of multiple genes via a single transformation event opens the possibility of expressing foreign pathways or pharmaceutical proteins involving multiple genes. Expressing the Cry2aA2 operon, including a putative chaperonin to aid in protein folding, in the chloroplast via a single transformation event leads to production of crystalized insecticidal proteins. Expressing the Mer operon via a single transformation event leads to a phytoremediation system.

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(54) **Title:** MULTIPLE GENE EXPRESSION FOR ENGINEERING NOVEL PATHWAYS AND HYPEREXPRESSION OF FOREIGN PROTEINS IN PLANTS

(57) **Abstract:** Introducing blocks of foreign genes in a single operon would avoid complications such as position effect and gene silencing inherent in putting one gene at a time into random locations in the nuclear genome. Cloning several genes into a single T-DNA does not avoid the compounded variable expression problem encountered in nuclear transgenic plants. This disclosure shows that a bacterial operon can be expressed in a single integration event as opposed to multiple events requiring several years to accomplish. Expression of multiple genes via a single transformation event opens the possibility of expressing foreign pathways or pharmaceutical proteins involving multiple genes. Expressing the Cry2aA2 operon, including a putative chaperonin to aid in protein folding, in the chloroplast via a single transformation event leads to production of crystalized insecticidal proteins. Expressing the Mer operon via a single transformation event leads to a phytoremediation system.

AMENDED CLAIMS

[received by the International Bureau on 21 August 2001 (21.08.01); original claims 1, 3, 5-7, 9, 10, 14, 16, 17, 19, 20, 22-25, 28, 32-37, 47 and 56 amended; new claim 61added remaining claims unchanged (7 pages)]

1. A stable chloroplast transformation and expression vector which is capable of introducing multiple genes in a multi-gene operon into a selected plant by a single integration event, wherein each step of said multiple genes is carried out by an enzyme encoding a heterologous DNA sequence which comprises an expression cassette, said vector comprising as operably linked components, in the 5' to the 3' direction of translation, a promoter operative in said plastids which drives the multi-gene operon, a selectable marker sequence, the multi-gene operon which is functional to co-express multiple enzymes in the plastids and is capable of functioning as a biosynthetic pathway, a transcription termination region functional in said plastids, and flanking each side of the expression cassette, flanking DNA sequences which are homomlogous to DNA sequences inclusive of a spacer sequence of the target plastid genome, whereby stable integration of the heterologous coding sequence into the chloroplast genome of the target plant is facilitated throughout homologous recombination of the flanking sequence with the homologous sequences in the target plastid gene.

2. A vector of claim 1, wherein a gene of the operon codes for an insecticidal toxin crystal protein.

3. A vector of claim 2, wherein the insecticidal toxin crystal protein is a *Bacillus thuringiensis* (Bt) crystal protein.

4. A vector of claim 3, wherein another gene of the operon codes for another insecticidal crystal protein with a different mode of action.

5. A vector of claim 4, wherein the multi-gene operon is functional to co-express, in addition to a Bt insecticidal toxin gene, a non-Bt insecticidal toxin gene selected from at least one of the group of cholesterol oxidase, alpha-amylate inhibitors, protease inhibitors, cowpea trypsin inhibitors and the potato proteinase inhibitor II, whereby gene pyramiding of the toxin product tends to retard the ability of insects to adapt to the insecticidal effect of the transgenic target plants.

6. A vector of claim 4 or 5, wherein one gene of said multi-gene operon codes for a putative chaperonin which facilitates the folding of the Bt crystal toxin protein to form proteotically stable cuboidal crystals.

7. A vector of claim 3, wherein the operon includes at least a gene encoding a Bt insecticidal crystal protein selected from the group consisting of *cry1Aa1, cry1Aa2,cry1Aa3,*

cry1Aa4, *cry1Aa5*, *cry1Aa6*, *cry1Ab1*, *cry1Ab2*, *cry1Ab3*, *cry1Ab4*, *cry1Ab5*, *cry1Ab6*, *cry1Ab7*, *cry1Ab8*, *cry1Ab9*, *cry1Ab10*, *cry1Ac1*, *cry1Ac2*, *cry1Ac3*, *cry1Ac4*, *cry1Ac5*, *cry1Ac6*, *cry1Ac7*, *cry1Ac8*, *cry1Ac9*, *cry1Ac10*, *cry1Ad1*, *cry1Ae1*, *cry1Af1*, *cry1Ba1*, *cry1Ba2*, *cry1Bb1*, *cry1Bc1*, *cry1Bd1*, *cry1Ca1*, *cry1Ca2*, *cry1Ca3*, *cry1Ca4*, *cry1Ca5*, *cry1Ca6*, *cry1Ca7*, *cry1Cb1*, *cry1Da1*, *cry1Db1*, *cry1Ea1*, *cry1Ea2*, *cry1Ea3*, *cry1Ea4*, *cry1Eb1*, *cry1Fa1*, *cry1Fa2*, *cry1Fb1*, *cry1Ga1*, *cry1Ga2*, *cry1Gb1*, *cry1Ha1*, *cry1Hb1*, *cry1Ia1*, *cry1Ia2*, *cry1Ia3*, *cry1Ia4*, *cry1Ia5*, *cry1Ib1*, *cry1Ja1*, *cry1Jb1*, *cry1Ka1*, *cry2Aa1*, *cry2Aa2*, *cry2Aa3*, *cry2Ab1*, *cry2Ab2*, *cry2Ac1*, *cry3Aa1*, *cry3Aa2*, *cry3Aa3*, *cry3Aa4*, *cry3Aa5*, *cry3Aa6*, *cry3Ba1*, *cry3Ba2*, *cry3Bb1*, *cry3Bb2*, *cry3Ca1*, *cry4Aa1*, *cry4Aa2*, *cry4Ba1*, *cry4Ba2*, *cry4Ba3*, *cry4Ba4*, *cry5Aa1*, *cry5Ab1*, *cry5Ac1*, *cry5Ba1*, *cry6Aa1*, *cry6Ba1*, *cry7Aa1*, *cry7Ab1*, *cry7Ab2*, *cry8Aa1*, *cry8Ba1*, *cry8Ca1*, *cry9Aa1*, *cry9Aa2*, *cry9Ba1*, *cry9Ca1*, *cry9Da1*, *cry9Da2*, *cry10Aa1*, *cry11Aa1*, *cry11Aa2*, *cry11Ba1*, *cry11Bb1*, *cry12Aa1*, *cry13Aa1*, *cry14Aa1*, *cry15Aa1*, *cry16Aa1*, *cry17Aa1*, *cry18Aa1*, *cry19Aa1*, *cry19Ba1*, *cry20Aa1*, *cry21Aa1*, *cry22Aa1*, *cyt1Aa1*, *cyt1Aa2*, *cyt1Aa3*, *cyt1Aa4*, *cyt1Ab1*, *cyt1Ba1*, *cyt2Aa1*, *cyt2Ba1*, and *cyt2Bb1*, wherein at least one of the genes of the operon codes for a Bt insecticidal crystal protein and another gene codes for a putative chaperonin which facilitates folding of the Bt protein to proteolytically stable cuboidal crystals.

8. A vector of claim 1, wherein at least one of the gene of the operon codes for a biopharmaceutical protein.

9. A vector of claim 7, wherein another gene of the operon codes for a putative chaperonin which facilitates folding of the protein.

10. A vector of claim 8, wherein the protein is selected from a group of insulin and human serum albumin.

11. A vector of claim 7, wherein another gene of the operon codes for another biopharmaceutical protein other than the gene which codes for the putative chaperonin, which protein is expressed in stoichimetric ratio.

12. A vector of claim 10, wherein the genes of the operon, other than the gene which codes for the putative chaperonin, codes for biopharmaceutical proteins which are expressed in stoichimetric ratio.

13. A vector of claims 7, 8, 9 or 10 which comprises collecting the protein product in a folded configuration, thereby enhancing their stability, and facilitating single step purification.

14. A method of combating insects which comprises applying to the insects or their habitat an insecticidally amount of the insecticidal crystal protein of claim 4.

15. A method of transforming a chloroplast of a selected plant species or the progeny thereof to confer insect resistance and producing on a large-scale foreign protein, said method comprising the steps of:

stably transforming the chloroplast of selected plant cells to express at least one insecticidal toxin protein and a chaperonin, growing the transformed plant cells under conditions which allow the expression of said insecticidal toxin protein and chaperonin.

16. The method of claim 15, further comprising the steps of culturing said plant cells in a plant growth medium comprising spectinomycin, and selecting transformed plant cells capable of growth in the presence of said spectinomycin.

17. The method of claim 16, further comprising regenerating a transformed plant from said transformed plant cells.

18. A transformed plant which has been transformed by the method of any one of claims 15-17.

19. The transformed plant of claim 18, wherein said plant contains a high accumulation of insecticidal toxin proteins in said plant's leaves.

20. The progeny of the transformed plant of claim 18.

21. A vector of claim 1, wherein the biosynthetic pathway is a bioremediation system that functions to degrade inorganic and organic metal compounds in contaminated sites.

22. A vector of claim 21, wherein the expression cassette does not contain a termination region.

23. A vector of claim 21 or claim 22, wherein the operon contains mercury resistance coding sequences encoding enzymes Mer A and Mer B.

24. The vector of claim 23, wherein the bioremediation system is driven by a single promoter.

25. The chloroplast transformation and expression vector of claim 24, wherein enzymes of the bioremediation system are expressed in stoichiometric amounts.

26. A vector of claim 25, wherein the inorganic compounds are selected from at least one of the group consisting of divalent cations of mercury, nickel, cobalt, trivalent cations of gold, and monovalent cations of silver.

27. A vector of claim 25, wherein the organic compounds are selected from at least one of the group consisting of alkyl mercury, alkenyl mercury, alkynyl mercury, aromatic mercury compounds, alkyl lead compounds, alkyl arsenic compounds and alkyl cadmium compounds.

28. A method of transforming a chloroplast of a selected plant species or the progeny thereof to confer greater resistance to metal ions than the corresponding parental plant which does not require several back crosses to create a complete pathway that detoxifies mercury and organiomercurial, said method comprising the steps of:

stably transforming the chloroplast of a plant by inserting an expression cassette containing the mercury resistance coding sequences of claim 21 into a plant species or the progeny thereof, growing the transforming plant species under conditions which allow the expression of said expression cassette.

29. The method of claim 28, further comprising culturing said plant in a plant growth medium comprising a selector for the corresponding selectable marker of claim 1, and selecting transformed plant cells capable of growth in the presence of said selector.

30. The method of claim 29, further comprising regenerating a transformed plant from said transformed plant cells.

31. A stably transformed plant which has been transformed by the methods of any one of claims 28-30.

32. The progeny of the stably transformed plant of claim 31.

33. A method of phytoremediation of mercury and organomercurials in soil and ground water, said method comprising the steps of:

planting the stably transformed plant of claim 31 or its progeny in soil contaminated with mercury and organomercurials and allowing said plants to grow.

34. A method of phytoremediation which does not require several back crosses to create a complete pathway that detoxifies mercury and organomercurials, said method comprising the methods of claim 33.

35. The plants formed by the method of claim 33, wherein the plant contains products of the bioremediation pathway.

36. The products of claim 35, wherein said products are metals that are reduced by the enzymes of the bioremediation pathway.

37. The vector of claim 23 which is capable of introducing a multiple-step biosymethtic pathway into a selected photosynthetic cell by a single integration event.

38. The vector of claim 37, wherein the biosynthetic pathway degrades inorganic and organic mercury compounds.

39. A vector of claim 38, wherein the bioremediation pathway is driven by a single promoter.

40. A vector of claim 38, wherein the enzymes of the bioremediation pathway are expressed in stoichiometric amounts.

41. A vector of claim 38, wherein the inorganic compounds are selected from at least one of a group consisting of divalent cations of mercury, nickel, cobalt, trivalent cations of gold, and monovalent cations of silver.

42. A vector of claim 38, wherein the organic compounds are selected from at least one of a group consisting of alkyl mercury, alkenyl mercury, alkynyl mercury, aromatic mercury compounds, alkyl lead compounds, alkyl arsenic compounds and alkyl cadmium compounds.

43. A photosynthetic organism transformed with the vector of claim 38 which is useful for bioremediation of mercury and organomercurial compounds from contaminated water bodies.

44. A method of transforming a chloroplast of a selected photosynthetic organism to confer greater resistance to metal ions, said method comprising the steps of:

stably transforming the chloroplast of a photosynthetic organism with the vector of claim 38, growing the transformed photosynthetic organism under conditions which allow the expression of said expression cassette.

45. The method of claim 44, further comprising culturing said photosynthetic organism in a growth medium comprising a selector, and selecting transformed cells capable of growth in the presence of said selector.

46. The method of claim 45, further comprising regenerating a transgenic photosynthetic organism from said transformed cells.

47. A method of phytoremediation of mercury and organomercurials in bodies of contaminated water, said method comprising the steps of:

treating water contaminated with mercury and organomercurials with the transgenic photosynthetic organism of claim 43 before releasing the water into the environment.

48. The photosynthetic organism of claim 43, wherein said photosynthetic organism is either a green algae or a cyanobacteria.

49. The photosynthetic organism of claim 48, wherein the green algae is *Chlorella vulgaris*.

50. The photosynthetic organism of claim 48, wherein the cyanobacteria is *Synechocytis*.

51. A vector of claim 1 wherein a multi-gene operon codes for a protein.

52. A vector of claim 51, wherein the protein is a biopharmaceutical protein.

53. A vector of claim 52, wherein the biopharmaceutical protein is a monoclonal antibody.

54. A vector of claim 53, wherein the protein is produced in the same stoichiometric ratio.

55. A vector of claim 4, wherein said another gene of the operon is selected from the group of cholesterol oxidase, alpha-amylase inhibitors, and proteinase inhibitors.

56. The vector of claim 1, wherein the promoter is a one functional in green or non-green plastids.

57. The promoter of claim 56, wherein said promoter is selected from the group of psbA, accD, or 16srRNA promoters.

58. The biosynthetic pathway of claim 1, wherein said biosynthetic pathway result in the production of compounds such as amino acids, fatty acids, carbohydrates, polymers, vitamins, antibiotics and dyes.

59. A vector of claim 8, where the protein is human serum albumin.

60. A vector of claim 1, which further comprises flanking each side of the expression cassette, flanking DNA sequences which are homologous to a DNA sequence inclusive of a spacer sequence of the target chloroplast genome, which sequence is conserved in the chloroplast genome of different plant species, whereby stable integration of the heterologous coding sequence into the chloroplast genome of the target plant is facilitated through homologous recombination of the flanking sequences with the homologous sequences in the target chloroplast genome.

61. A plant or a seed of a plant which comprises at least one chloroplast which genome contains a promoter which drives a multi-gene operon, a selectable marker sequence,

and a multi-gene operon which is functional to co-express multiple enzymes which is capable of functioning as a biosynthetic pathway.

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(54) Title: METHODS FOR TRANSFORMING PLANTS TO EXPRESS BACILLUS THURINGIENSIS DELTA-ENDOTOXINS

(57) Abstract

Disclosed is a means of controlling plant pests by a novel method of expressing Cry2A *B. thuringiensis* δ-endotoxins in plants. The invention comprises novel nucleic acid segments encoding proteins comprising Cry2A *B. thuringiensis* δ-endotoxins. The nucleic acid segments are disclosed, as are transformation vectors containing the nucleic acid segments, plants transformed with the claimed segments, methods for transforming plants, and methods of controlling plant infestation by pests.

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

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WRITTEN OPINION

(PCT Rule 66)

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AUBURN UNIVERSITY

1. This written opinion is the first (first, etc.) drawn by this International Preliminary Examining Authority.

2. This opinion contains indications relating to the following items:

I	[X]	Basis of the opinion
II	[ ]	Priority
III	[X]	Non-establishment of opinion with regard to novelty, inventive step or industrial applicability
IV	[X]	Lack of unity of invention
V	[X]	Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	[ ]	Certain documents cited
VII	[X]	Certain defects in the international application
VIII	[X]	Certain observations on the international application

3. The applicant is hereby invited to reply to this opinion.

When? See the time limit indicated above. ~~The applicant may, before the expiration of that time limit, request this Authority to grant an extension., see Rule 66.2(d).~~

How? By submitting a written reply, accompanied, where appropriate, by amendments, according to Rule 66.3. For the form and the language of the amendments, see Rules 66.8 and 66.9.

Also For an additional opportunity to submit amendments, see Rule 66.4.
For the examiner's obligation to consider amendments and/or arguments, see Rule 66.4 *bis*.
For an informal communication with the examiner, see Rule 66.6.

If no reply is filed, the international preliminary examination report will be established on the basis of this opinion.

4. The final date by which the international preliminary examination report must be established according to Rule 69.2 is: 29 JUNE 2002.

Facsimile No.	Authorized officer AND Telephone No.
(703) 305-3230	ANNE R. KUBELIK (703) 308-0196

WRITTEN OPINION
Form PCT/IPEA/408 (Box I) (July 1998)
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International application No.
PCT/US01/06276

I. Basis of the opinion

1. With regard to the **elements** of the international application:*

[X] the international application as originally filed

[X] the description:
pages 1-45 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the claims:
pages 46-51 , as originally filed
pages NONE , as amended (together with any statement) under Article 19
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the drawing:
pages 1-14 , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

[X] the sequence listing part of the description:
pages NONE , as originally filed
pages NONE , filed with the demand
pages NONE , filed with the letter of _____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

[ ] the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

[ ] the language of publication of the international application (under Rule 48.3(b)).

[ ] the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the written opinion was drawn on the basis of the sequence listing:

[ ] contained in the international application in printed form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[ ] furnished subsequently to this Authority in computer readable form.

[ ] The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[ ] The statement that the information recorded in computer readable form is identical to the writen sequence listing has been furnished.

4. [X] The amendments have resulted in the cancellation of:

[X] the description, pages NONE

[X] the claims, Nos. NONE

[X] the drawings, sheets/~~fig~~ NONE

5. [ ] This opinion has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).

** Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this opinion as "originally filed".*

WRITTEN OPINION
Form PCT/IPEA/408 (Box II) (July 1998)
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International application No.
PCT/US01/06276

II. Priority

1. ☐ This opinion has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

   ☐ copy of the earlier application whose priority has been claimed.

   ☐ translation of the earlier application whose priority has been claimed.

2. ☐ This opinion has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

Thus for the purposes of this opinion, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:

WRITTEN OPINION

Form PCT/IPEA/408 (Box III) (July 1998)

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International application No.

PCT/US01/06276

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been and will not be examined in respect of:

☐ the entire international application.

☒ claims Nos. 7, 9, 11, 13, 33-36, 56-57

because:

☐ the said international application, or the said claim Nos. _ relate to the following subject matter which does not require international preliminary examination *(specify)*.

☐ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. _ are so unclear that no meaningful opinion could be formed *(specify)*.

☐ the claims, or said claims Nos. _ are so inadequately supported by the description that no meaningful opinion could be formed.

☒ no international search report has been established for said claims Nos. (See Attached) .

2. A written opinion cannot be drawn due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

WRITTEN OPINION

Form PCT/IPEA/408 (Box IV) (July 1998)
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International application No.
PCT/US01/06276

IV. Lack of unity of invention

1. In response to the invitation (Form PCT/IPEA/405) to restrict or pay additional fees the applicant has:

   ☐ restricted the claims. (SEE SUPPLEMENTAL SHEET)

   ☒ paid additional fees.

   ☐ paid additional fees under protest.

   ☐ neither restricted nor paid additional fees.

2. This Authority found that the requirement of unity of invention is not complied with for the following reasons and chose, according to Rule 68.1 not to invite the applicant to restrict or pay additional fees:

3. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this opinion:

   ☐ all parts.

   ☒ the parts relating to claims Nos. 1-6, 8, 10, 12, 14-32, 37-55, 58-60.

WRITTEN OPINION

Form PCT/IPEA/408 (Box V) (July 1998)
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International application No.
PCT/US01/06276

V. Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. statement

Novelty (N)	Claims	(Please See supplemental sheet)	YES
	Claims	(Please See supplemental sheet)	NO
Inventive Step (IS)	Claims	(Please See supplemental sheet)	YES
	Claims	(Please See supplemental sheet)	NO
Industrial Applicability (IA)	Claims	(Please See supplemental sheet)	YES
	Claims	(Please See supplemental sheet)	NO

2. citations and explanations

Claims 1-6, 8, 10, 12, 14-32, 37-55, and 58-60 meet the criteria set out in PCT Article 33 (4) because plastid transformation vectors, methods of transforming plastids and plants with transformed plastids have industrial applicability.

Claims 4-6, 10, 12, 15-20, 24-32, 39-42, 44-47, 49, 53-55 and 59 meet the criteria set out in PCT Article 33(2), because the prior art does not teach plastid transformation vectors encoding a Bt crystal protein and a chaperonin, or a Bt crystal protein and a non-Bt toxin, or insulin, nor does it teach plants whose plastids are transformed with those vectors. The prior art also does not teach eukaryotic plants whose plastids have been transformed with mercury resistance genes.

Claims 1, 51, 58 and 60 lack novelty under PCT Article 33(2) as being anticipated by MALIGA et al (US 5,877,402, 1999). Maliga et al teach a plastid transformation vector that comprises a promoter driving a multi-gene operon, a selectable marker sequence, a multi-gene operon encoding a protein, a transcription termination region, and flanking regions homologous to the target plastid genome. Maliga et al also teach methods of plastid transformation (Fig. 30, column 65, lines 19-39, and column 66, lines 16-39). Some of the plastid transformation vectors produce dye because they have the beta-galactosidase gene.

Claims 1-3, 14, 51 and 60 lack novelty under PCT Article 33(2) as being anticipated by MCBRIDE et al (US 5,545,818, 1996). McBride et al teach plastid transformation vectors that encode Bacillus thuringiensis (Bt) crystal proteins (column 6, lines 3-15, and column 7, line 53, to column 8, line 5). McBride et al also teach a method of controlling insects by the application of a crystal protein to their environment (column 10, line 1-60).

Claims 1-3, 8, 51-52 and 60 lack novelty under PCT Article 33(2) as being anticipated by CALGENE LLC (WO 00/03012). Calgene LLC teaches plastid transformation vectors encoding the biopharmaceutical proteins aprotinin and human growth hormone (pages 8-9, 23-24, and 37-40) and ones encoding Bacillus (Continued on Supplemental Sheet.)

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International application No.
PCT/US01/06276

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

Application No. Patent No.	Publication Date *(day/month/year)*	Filing Date *(day/month/year)*	Priority date (valid claim) *(day/month/year)*

2. Non-written disclosures (Rule 70.9)

Kind of non-written disclosure	Date of non-written disclosure *(day/month/year)*	Date of written disclosure referring to non-written disclosure *(day/month/year)*

WRITTEN OPINION

Form PCT/IPEA/408 (Box VII) (July 1998)
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International application No.

PCT/US01/06276

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

Claims 1, 3 and 37 are objected to under PCT Rule 66.2(a)(iii) as containing the following defect(s) in the form or contents thereof:

Misspellings were noted of "homomlogous" in claim 1, "thumngensis" in claim 3, and "biosymethtic" in claim 37.

International application No.
PCT/US01/06276

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

The description is objected to under PCT Rule 66.2(a)(v) as lacking clarity under PCT Article 5 because it fails to adequately enable practice of the claimed invention because: cyanobacteria like Synechocytis do not have plastids, yet claim 50 requires that Synechocytis be transformed with a plastid transformation vector.

Claims 1, 21-23, 37-38, 43, 48 and 50 are objected to as lacking clarity under PCT Rule 66.2(a)(v) because practice of the claimed invention is not enabled as required under PCT Rule 5.1(a) for the reasons set forth in the immediately preceding paragraph.

The description is objected to under PCT Rule 66.2(a)(v) as lacking clarity under PCT Article 5 because it fails to contain an adequate written description of DNA sequences for homologous integration into the Synechocytis genome. The description is inadequate because: such sequences are neither presented in the description nor cited in a reference.

Claims 1, 21-23, 37-38, 43, 48 and 50 are objected to as lacking clarity under PCT Rule 66.2(a)(v) because practice of the claimed invention is not adequately described in writing, as required under PCT Rule 5.1(a)(iii), for the reasons set forth in the immediately preceding paragraph.

Claims 12, 16, 18 and 54 are objected to under PCT Rule 66.2(a)(v) as lacking clarity under PCT Article 6 because the claims are indefinite for the following reason(s):
Claim 14 is drawn to a method of controlling an insect by topical application of a protein, while dependent claim 16 is drawn to a method using s step of culturing plant cells. For purposes of examination, claim 16 was treated as though it were dependent upon claim 15, which is a method of transforming the chloroplast of a plant. Similarly, dependent claim 18 is drawn to a transformed plant of any one of claims 14-16. As claim 14 is not drawn to a transformed plant, for purposes of examination, claim 18 was treated as though it were dependent upon either claim 15 or 16.

Claim 12 recites the limitation "the putative chaperonin" in line 2. There is insufficient antecedent basis for this limitation in the claim.

Claim 21 recites the limitation "the biosynthetic pathway" in line 1. There is insufficient antecedent basis for this limitation in the claim.

In claim 54 it is not clear what the protein is in the same (Continued on Supplemental Sheet.)

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

TIME LIMIT:

The time limit set for response to a Written Opinion may not be extended. 37 CFR 1.484(d). Any response received after the expiration of the time limit set in the Written Opinion will not be considered in preparing the International Preliminary Examination Report.

CLASSIFICATION:

The International Patent Classification (IPC) and/or the National classification are as listed below:
IPC(7): A01H 5/02, 5/10, 13/00; C12N 1/20, 1/12, 5/04, 15/29, 15/32, 15/52, 15/31, 15/17, 15/13, 15/63, 15/74, 15/82 and
US Cl.: 435/320.1, 468, 252.3, 430, 418, 419; 536/23.71, 23.5, 23.6, 23.7, 23.2, 23.53; 800/278, 279, 288, 298, 302, 298, 287; 514/2

III. NON-ESTABLISHMENT OF OPINION:

No international search report has been established for claim numbers 7, 9, 11, 13, 33-36, 56-57.

IV. LACK OF UNITY OF INVENTION:

1. This response is made to a telephone Lack of Unity requirement (see telephone memorandum attached hereto or attached to a prior Written Opinion).

V. 1. REASONED STATEMENTS:
The opinion as to Novelty was positive (YES) with respect to claims 4-6, 10, 12, 15-20, 24-32, 39-42, 44-47, 49, 53-55 and 59.
The opinion as to Novelty was negative (NO) with respect to claims 1-3, 8, 14, 21-23, 37-38, 43, 48, 50-52, 58 and 60.
The opinion as to Inventive Step was positive (YES) with respect to claims NONE.
The opinion as to Inventive Step was negative (NO) with respect to claims 1-6, 8, 10, 12, 14-32, 37-55 and 58-60.
The opinion as to Industrial Applicability was positive (YES) with respect to claims 1-6, 8, 10, 12, 14-32, 37-55 and 58-60.
The opinion as to Industrial Applicability was negative (NO) with respect to claims NONE.

V. 2. REASONED STATEMENTS - CITATIONS AND EXPLANATIONS (Continued):
thuringiensis (Bt) crystal proteins (pg 21).

Claims 1, 51, 58 and 60 lack novelty under PCT Article 33(2) as being anticipated by KHAN et al (1999, Nature Biotechnol. 17:910-915).
Khan et al teach plastid transformation vectors encoding a fusion protein between a green fluorescent protein and an antibiotic resistance protein (entire article).

Claims 1-3, 14, 51, and 60 lack novelty under PCT Article 33(2) as being anticipated by KOTA et al (1999, Proc. Natl. Acad. Sci. USA 96:1840-1845).
Kota et al teach a plastid transformation vector encoding a Bacillus thuringiensis (Bt) crystal protein (pg 1842). Kota et al also teach a method of controlling insects by the application of a crystal protein to their environment (pg 1843).

Claims 1, 51, 58 and 60 lack novelty under PCT Article 33(2) as being anticipated by DANIELL et al (US 6,004,782, 1999).
Daniell et al teach plastid transformation vectors encoding the polymer poly(VPGVP) (column 20, line 31, to column 21, line 6).

Claim 14 lacks novelty under PCT Article 33(2) as being anticipated by FUCHS et al (US 5,250,515, 1993).
Fuchs et al teach a method of controlling insects by the application of a crystal protein to their environment (column 28, lines 19-31, and claims 1-4).

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

Claims 1-4, 14, 51 and 60 lack an inventive step under PCT Article 33(3) as being obvious over each of MCBRIDE et al (US 5,545,818, 1996) and KOTA et al (1999, Proc. Natl. Acad. Sci. USA 96:1840-1845). in view of CRICKMORE et al (1998, Microbiol. Mol. Biol. Rev. 62:807-813).
The claims are drawn to plastid transformation vectors encoding a variety of Bt crystal protein genes.
McBride et al teach plastid transformation vectors that encode Bt crystal proteins and methods of plastid transformation (column 6, lines 3-15, and column 7, line 53, to column 8, line 5). McBride et al do not disclose a variety of Bt crystal protein genes.
Kota et al teach a plastid transformation vector encoding a Bt crystal protein and methods of plastid transformation (pg 1842).
Kota et al do not teach a variety of Bt crystal protein genes.
Crickmore et al teach a variety of Bt crystal protein genes.
At the time the invention was made, it would have been obvious to one of ordinary skill in the art to produce plants transformed with plastid transformation vectors encoding a Bt crystal protein gene as taught by each of McBride et al and Kota et al, and to modify that to express other or multiple Bt crystal protein genes as described in Crickmore et al. One of ordinary skill in the art would have been motivated to do so because of the advantages of plastid expression of Bt crystal proteins (Kota et al pg 1844).

Claims 1-6, 14-20, 51 and 60 lack an inventive step under PCT Article 33(3) as being obvious over the prior art as applied in the immediately preceding paragraph and further in view of BOSTON et al (1996, Plant Mol. Bio. 32:191-222).
The claims are drawn to plastid transformation vectors encoding a Bt crystal protein and a chaperonin, methods of plastid transformation with those vectors and plants transformed with them.
Each of McBride et al and Kota et al in view of Crickmore et al disclose plastid transformation vectors encoding a variety of Bt crystal protein genes and plants transformed with those vectors. Each of McBride et al and Kota et al in view of Crickmore et al do not disclose plastid transformation vectors encoding a Bt crystal protein and a chaperonin.
Boston et al teach the importance of chaperonins in plastid protein folding and that the gene for chaperonin is plastid-encoded in some plants (pg 204-207 and 209).
At the time the invention was made, it would have been obvious to one of ordinary skill in the art to produce plants transformed with plastid transformation vectors encoding a variety of Bt crystal protein genes as taught by each of McBride et al and Kota et al in view of Crickmore et al, and to modify that to also express chaperonins in those constructs as described in Boston et al. One of ordinary skill in the art would have been motivated to do so because of the importance of chaperonins in plastid gene expression (Boston et al, pg 204-207).

Claims 1, 51, 58 and 60 lack an inventive step under PCT Article 33(3) as being obvious over MALIGA et al (US 5,877,402, 1999) in view of GRUYS et al (US 5,958,745, 1999).
The claims are drawn to plastid transformation vectors encoding an amino acid biosynthetic pathway.
Maliga et al disclose teach a plastid transformation vector that comprises a promoter driving a multi-gene operon, a selectable marker sequence, a multi-gene operon encoding a protein, a transcription termination region and flanking regions homologous to the target plastid genome and methods of plastid transformation (Fig. 30, column 65, lines 19-39, and column 66, lines 16-39).
Maliga et al suggest putting gene clusters encoding complex biosynthetic pathways into plastid transformation vectors (column 27, lines 14-59; column 65, lines 28-39). Maliga et al do not teach genes encoding those pathways.
Gruys et al teach plants transformed with genes of the E. coli isoleucine biosynthetic pathway; these genes are targeted to the chloroplast via transit peptides (column 69, line 9, to column 72, line 18).
At the time the invention was made, it would have been obvious to one of ordinary skill in the art to transform plant plastids as taught by Maliga et al, and to modify that to use genes encoding amino acid biosynthesis proteins as described in Gruys et al. One of ordinary skill in the art would have been motivated to do so because of the suggestion of Maliga et al to do so (column 65, lines 28-39) and because selection of transformation of the chloroplast by transformation with a plastid transformation vector versus transformation using chloroplast transit peptides is an obvious design choice.

Claims 1, 51, 58 and 60 lack an inventive step under PCT Article 33(3) as being obvious over MALIGA et al (US 5,877,402, 1999) in view of PATTON (US 5,869,719, 1999).
The claims are drawn to plastid transformation vectors encoding a vitamin biosynthetic pathway.
Maliga et al disclose teach a plastid transformation vector that comprises a promoter driving a multi-gene operon, a selectable marker sequence, a multi-gene operon encoding a protein, a transcription termination region and flanking regions homologous to the target plastid genome and methods of plastid transformation (Fig. 30, column 65, lines 19-39, and column 66, lines 16-39).
Maliga et al suggest putting gene clusters encoding complex biosynthetic pathways into plastid transformation vectors (column

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

27, lines 14-59; column 65, lines 28-39). Maliga et al do not teach genes encoding those pathways.
Patton teaches plants transformed with biotin biosynthetic genes (column 22, line 5, to column 24, line 25).
At the time the invention was made, it would have been obvious to one of ordinary skill in the art to transform plant plastids as taught by Maliga et al, and to modify that to use genes encoding amino acid biosynthesis proteins as described in Patton. One of ordinary skill in the art would have been motivated to do so because of the suggestion of Maliga et al to do so (column 65, lines 28-39), because of the suggestion of Patton to do so (column 9, lines 37-44), and because selection of transformation of the chloroplast by transformation with a plastid transformation vector versus transformation using chloroplast transit peptides is an obvious design choice.

Claims 1, 8, 10, 12, 51-52 and 60 lack an inventive step under PCT Article 33(3) as being obvious over CALGENE LLC (WO 00/03012) in view of CARRINGTON et al (US 5,766,885, 1998).
The claims are drawn to plastid transformation vectors encoding insulin.
Calgene LLC teaches plastid transformation vectors encoding the biopharmaceutical proteins aprotinin and human growth hormone (pages 8-9, 23-24, and 37-40). Calgene LLC suggest expressing an insulin gene in plant plastids (pg 8, lines 31-33), but do not disclose an insulin gene.
Carrington et al teach plants transformed with a gene encoding human insulin (column 10, lines 53-67, column 20, lines 30-36, and claim 8).
At the time the invention was made, it would have been obvious to one of ordinary skill in the art to express biopharmaceutical proteins in plant plastids as taught by Calgene LLC, and to modify that to make insulin that protein as described in Carrington et al. One of ordinary skill in the art would have been motivated to do so because of the suggestion of Calgene LLC to do so (pg 8, lines 31-33).

Claims 1, 8, 51-54 and 60 lack an inventive step under PCT Article 33(3) as being obvious over CALGENE LLC (WO 00/03012) in view of JOHN INNES CENTRE (WO 99/66026).
The claims are drawn to plastid transformation vectors encoding antibodies.
Calgene LLC teaches plastid transformation vectors encoding the biopharmaceutical proteins aprotinin and human growth hormone (pages 8-9, 23-24, and 37-40). Calgene LLC suggest expressing monoclonal antibodies in plant plastids (claims 11 and 21), but do not disclose an antibody gene.
John Innes Centre teaches plants transformed with genes encoding antibodies (pg 23-56). These antibodies would be monoclonal.
At the time the invention was made, it would have been obvious to one of ordinary skill in the art to express biopharmaceutical proteins in plant plastids as taught by Calgene LLC, and to modify that to make an antibody as that protein as described in John Innes Centre. One of ordinary skill in the art would have been motivated to do so because the suggestion of Calgene LLC to do so (claims 11 and 21) and because John Innes Centre suggests targeting the expression of the antibody gene to the chloroplast (pg 8).

Claims 1, 8, 51-52 and 59-60 lack an inventive step under PCT Article 33(3) as being obvious over CALGENE LLC (WO 00/03012) in view of SIJMONS et al (1990, Biotechnology 8:217-221).
The claims are drawn to plastid transformation vectors encoding human serum albumin.
Calgene LLC teaches plastid transformation vectors encoding the biopharmaceutical proteins aprotinin and human growth hormone (pages 8-9, 23-24, and 37-40). Calgene LLC do not disclose plastid transformation vectors encoding human serum albumin.
Sijmons et al teach the production of correctly processed human serum albumin in transgenic plants (pg 217-218).
At the time the invention was made, it would have been obvious to one of ordinary skill in the art to express biopharmaceutical proteins in plant plastids as taught by Calgene LLC, and to modify that to express human serum albumin as described in Sijmons et al. One of ordinary skill in the art would have been motivated to do so because the substitution of one biopharmaceutical protein for another is an obvious design choice.

Claims 1-5, 51, 55 and 60 lack an inventive step under PCT Article 33(3) as being obvious over MCBRIDE et al (US 5,545,818 A, 1996) in view of GREENPLATE et al (US 5,763,245, 1998).
The claims are drawn to vectors for transformation of a plant plastid with both a cholesterol oxidase gene and a B. thuringiensis crystal protein gene.

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Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

McBride et al teach plastid transformation vectors that encode B. thuringiensis crystal proteins (column 6, lines 3-15, and column 7, line 53, to column 8, line 5). McBride et al also teach a method of controlling insects by the application of a crystal protein to their environment (column 10, line 1-60). McBride et al do not disclose transformation of a plant with both a cholesterol oxidase gene and a B. thuringiensis crystal protein gene.
Greenplate et al teach plants transformed with a cholesterol oxidase gene in combination with a B. thuringiensis crystal protein gene (column 3, lines 3-9, and claims 11-18). Greenplate et al also suggest expression of these genes in the chloroplast (column 18, line 48-63).
At the time the invention was made, it would have been obvious to one of ordinary skill in the art to transform plastid with a vector encoding a B. thuringiensis crystal protein gene as taught by McBride et al, and to modify that to add the cholesterol oxidase gene as described in Greenplate et al. One of ordinary skill in the art would have been motivated to do so because of the suggestion of Greenplate et al to express these genes in the chloroplast (column 18, line 48-63).

Claims 1-5, 51, 55 and 60 lack an inventive step under PCT Article 33(3) as being obvious over the prior art as applied in the immediately preceding paragraph and further in view of each of CHEN et al (US 5,981,722, 1999) and THOMAS et al (US 5,436,392, 1995).
The claims are drawn to plastid transformation encoding both a proteinase inhibitor gene and a B. thuringiensis crystal protein gene.
McBride et al in view of Greenplate et al disclose plastid transformation encoding both a cholesterol oxidase gene and a B. thuringiensis crystal protein gene. McBride et al in view of Greenplate et al do not disclose vectors with proteinase inhibitor gene and a B. thuringiensis crystal protein gene.
Chen et al teach plants transformed with a trypsin inhibitor gene (claims 1-21).
Thomas et al teach plants transformed with a proteinase inhibitor gene (claims 1-16).
At the time the invention was made, it would have been obvious to one of ordinary skill in the art to transform plastids with vectors comprising two insect toxin genes as taught by McBride et al in view of Greenplate et al, and to modify that to use a proteinase inhibitor as one of those toxin genes as described in each of Chen et al and Thomas et al. One of ordinary skill in the art would have been motivated to do so because substitution of one insect toxin gene for another is an obvious design choice.

Claims 1, 21-32, 37-47, 51 and 60 lack an inventive step under PCT Article 33(3) as being obvious over MALIGA et al (US 5,877,402, 1999) in view of MEAGHER et al (US 5,965,796, 1999).
The claims are drawn to plastid transformation vectors encoding MerA and MerB, plants and seeds transformed with that vector, and methods of plastid transformation.
Maliga et al teach a plastid transformation vector that comprises a promoter driving a multi-gene operon, a selectable marker sequence, a multi-gene operon encoding a protein, a transcription termination region, and flanking regions homologous to the target plastid genome. Maliga et al also teach methods of plastid transformation (Fig. 30, column 65, lines 19-39, and column 66, lines 16-39). Maliga et al suggest putting gene clusters encoding complex biosynthetic pathways into plastid transformation vectors (column 27, lines 14-59; column 65, lines 28-39). Maliga et al do not disclose genes encoding MerA or MerB.
Meagher et al teach plants and their seeds transformed with genes encoding MerA and MerB (column 3, lines 31-45; column 3, line 58, to column 4, line 20, column 21, line 50, to column 25, line 53)
At the time the invention was made, it would have been obvious to one of ordinary skill in the art to transform plant plastids with multiple genes as taught by Maliga et al, and to modify that to express MerA and MerB as described in Meagher et al. One of ordinary skill in the art would have been motivated to do so because of the suggestion of Maliga et al to express a wide variety of genes in plastids (column 27, lines 14-59; column 65, lines 28-39).

Claims 1, 21-23, 37-38, 43, 48 and 50 lack novelty under PCT Article 33(2) as being anticipated by ROSSEN (US 5,571,722, 1996).
Rossen teaches photosynthetic bacteria transformed with the MerA and MerB genes (column 15, line 20, to column 18, line 42).

Claims 1, 21-23, 37-38, 43, 48-51 and 60 lack an inventive step under PCT Article 33(3) as being obvious over DUNAHAY et al (US 5,661,017, 1997) in view of ROSSEN (US 5,571,722, 1996).
The claims are drawn to Chlorella whose plastids have been transformed with genes encoding MerA and MerB.
Dunahay et al teach the transformation of the plastids of a variety of algae (claims 1-2 and 21) and suggest their use in bioremediation (column 1, lines 22-33). Dunahay et al do not disclose transformation of algal plastids with genes encoding

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Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

MerA and MerB.
Rossen teaches photosynthetic bacteria transformed with the MerA and MerB genes and methods of phytoremediation (column 15, line 20, to column 18, line 42).
At the time the invention was made, it would have been obvious to one of ordinary skill in the art to transform algae plastids as taught by Dunahay et al, and to modify that to express MerA and MerB as described in Rossen. One of ordinary skill in the art would have been motivated to do so because of the overall utility in using single-celled photosynthetic organisms in bioremediation.

---------- NEW CITATIONS ----------

SIJMONS et al. Production of correctly processed human serum albumin in transgenic plants. Biotechnology. March 1990, Vol. 8, No. 3, pages 217-221, see pages 217-218.

VIII. CERTAIN OBSERVATIONS ON THE APPLICATION (Continued):

stoichiometric ratio to.

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A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/320.1, 468, 252.3, 430, 418, 419; 536/23.71, 23.5, 23.6, 23.7, 23.2, 23.53; 800/278, 279, 288, 298, 302, 298, 287; 514/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	US 5,877,402 A (MALIGA et al) 02 March 1999, col. 27, line 14 to col. 28, line 10; Figures 22 and 30, col. 65, lines 19-39; col. 66, lines 16-39.	1, 51, 58, 60 2-6, 8, 10, 12, 15-32, 37-49, 52-55, 59
X Y	US 5,545,818 A (MCBRIDE et al) 13 August 1996, col. 1, lines 55-64; col. 6, lines 3-15, col. 7, line 53 to col. 8, line 5.	1-3 4-6, 15-20, 55
X Y	WO 00/03012 A2 (CALGENE LLC) 20 January 2000, pages 2, 8-9, 21,and 23-24.	1-3, 8, 51-52 10, 12, 53-54

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
28 MAY 2001	21 JUN 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ANNE R. KUBELIK Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)☆

INTERNATIONAL SEARCH REPORT

International application No.
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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	KHAN et al. Fluorescent antibiotic resistance marker for tracking plastid transformation in higher plants. Nature Biotechnol. September 1999, Vol. 17, pages 910-915, see entire article.	1, 51, 58, 60 2-6, 8, 10, 12, 15-32, 37-49, 52-55, 59
X Y	KOTA et al. Overexpression of the Bacillus thuringiensis (Bt) Cry2Aa2 protein in chloroplasts confers resistance to plants against susceptible and Bt-resistant insects. Proc. Natl. Acad. Sci. USA March 1999, Vol. 96, pages 1840-1845, see pages 1842 and 1844.	1-5, 51, 55, 60 6, 15-20
X	US 6,004,782 A (DANIELL et al) 21 December 1999, col. 20, line 31 to col. 21, line 6.	1, 51, 58, 60
Y	US 5,958,745 A (GRUYS et al) 28 September 1999, col. 105, line 13, to col. 106, line 49; col. 98, lines 7-28.	1, 51, 58, 60
Y	CRICKMORE et al. Revision of the Nomenclature for Bacillus thuringiensis Pesticidal Crystal Proteins. Microbiol. Molec. Biol. Rev. September 1998, Vol. 62, No. 3, pages 807-813, see Table 1.	2-6, 15-20, 55
Y	BOSTON et al. Molecular chaperones and protein folding in plants. Plant Molec. Biol. 1996, Vol. 32, pages 191-222, see pages 204-207 and 209.	6, 12, 15-20
Y	US 5,869,719 A (PATTON, D.A.) 09 February 1999, col. 9, lines 37-44; col. 16, line 48-63.	1, 51, 58, 60
Y,P	US 6,140,486 A (FACCIOTTI et al) 31 October 2000, col. 15, lines 42-67; col. 27, lines 4-19.	1, 51, 58, 60
Y	US 5,436,392 A (THOMAS et al) 25 July, 1995, col. 1, lines 52-68.	4-5, 55
Y	US 5,763,245 A (GREENPLATE et al) 09 June 1998, col. 3, lines 3-9; col. 18, lines 48-63.	4-5, 55
Y	US 5,981,722 A (CHEN et al) 09 November 1999, col. 11, lines 16-27; col. 12, 42-54.	4-5, 55
Y	US 5,965,796 A (MEAGHER et al) 12 October 1999, col. 3, lines 31-45; col. 3, line 58 to col. 4, line 20.	21-32, 37-48

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,766,885 A (CARRINGTON et al) 16 June 1998, col. 10, lines 53-67.	8, 10, 12, 51-52
Y	WO 99/66026 A2 (JOHN INNES CENTRE) 23 December 1999, pages 1, 8 and 46-47, claim 10.	1, 8, 51-54
X	US 5,571,722 A (ROSSON R.A.) 05 November 1996, col. 15, lines 24-35; col. 18, lines 11-42.	50
Y		43, 48-49
Y	US 5,661,017 A (DUNAHAY et al) 26 August 1997, col. 12, line 8 to col. 13, line 29.	48-49
X	US 5,250,515 A (FUCHS et al) 05 October 1993, col. 28, lines 19-31; claims 1-4.	14
X,P	WO 01/07590 A2 (SYNGENTA PARTICIPATIONS AG) 01 February 2001 pages 1-2.	1, 51, 58, 60
X,P	DE COSA et al. Overexpression of the Bt cry2Aa2 operon in chloroplasts leads to formation of insecticidal crystals. Nature Biotechnol. January 2001, Vol. 19, pages 71-74, see entire article.	1-4, 6, 15-20

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Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 7, 9, 11, 13 and 56-57
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Claim 7, upon which the others are dependent, refers to genes in a publication. PCT Rule 6.2(a) states that the claims should not refer to the disclosure. Additionally, the publication is incorrectly cited.

3. ☒ Claims Nos.: 33-36 and 56-57
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/06276

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (7):

A01H 5/02, 5/10, 13/00; C12N 1/20, 1/12, 5/04, 15/29, 15/32, 15/52, 15/31, 15/17, 15/13, 15/63, 15/74, 15/82

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/320.1, 468, 252.3, 430, 418, 419; 536/23.71, 23.5, 23.6, 23.7, 23.2, 23.53; 800/278, 279, 288, 298, 302, 298, 287; 514/2

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

AGRICOLA, BIOSIS, CAPLUS, USPAT, JPO, EPO, DERWENT
Search terms; (plastid or chloroplast) transform?, insulin, biotin, albumin, antibody, mercury, merA, merB, algae, Bt, alpha amylase inhibitor, (protease or proteinase) inhibitor, trypsin inhibitor, cholesterol oxidase, fatty acid, polymer

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1.

Group I, claim(s) 1-4, 6, 15-20, 51 and 60, drawn to a chloroplast transformation vector encoding a Bt toxin and a chaperonin.

Group II, claim(s) 1, 4-6, 51, 55 and 60, drawn to a chloroplast transformation vector encoding a Bt toxin and a cholesterol oxidase.

Group III, claim(s) 1, 4-6, 51, 55 and 60, drawn to a chloroplast transformation vector encoding a Bt toxin and an alpha-amylate inhibitor.

Group IV, claim(s) 1, 4-6, 51, 55 and 60, drawn to a chloroplast transformation vector encoding a Bt toxin and a protease inhibitor.

Group V, claim(s) 1, 4-6, 51, 55 and 60, drawn to a chloroplast transformation vector encdoing a Bt toxin and a cowpea typrsin inhibitor.

Group VI, claim(s) 1, 4-6, 51, 55 and 60, drawn to a chloroplast transformation vector encoding a Bt toxin and a potato proteinase inhibitor II.

Group VII, claim(s) 1, 8, 10, 12 and 51-52, drawn to a chloroplast transformation vector encoding insulin.

Group VIII, claim(s) 1, 8, 10, 12, 51-52 and 59, drawn to a chloroplast transformation vector encoding human albumin.

Group IX, claim(s) 14, drawn to a method of combatting insects via application of proteins.

Group X, claim(s)1, 21-32, 37-42, 51 and 60, drawn to a plant chloroplast transformation vector comprising MerA and MerB, a method of transforming a chloroplast with that vector to confer resistance to metal ions, and a plant so obtained.

Group XI, claim(s) 1, 23 and 37-51, drawn to a chloroplast transformation vector comprising MerA and MerB for transformation of a single-celled organism, a method of transformation, a method of phytoremediation, and transformed algae and cyanobacteria.

Group XII, claim(s) 1, 51-54 and 60, drawn to a chloroplast transformation vector encoding a monoclonal antibody.

Group XIII, claim(s) 1, 51, 58 and 60, drawn to a chloroplast transformation vector encoding an amino acid biosynthetic pathway.

Group XIV, claim(s) 1, 51, 58 and 60, drawn to a chloroplast transformation vector encoding an fatty acid biosynthetic pathway.

Group XV, claim(s) 1, 51, 58 and 60, drawn to a chloroplast transformation vector encoding a carbohydrate biosynthetic pathway.

Group XVI, claim(s) 1, 51, 58 and 60, drawn to a chloroplast transformation vector encoding a polymer biosynthetic pathway.

Group XVII, claim(s) 1, 51, 58 and 60, drawn to a chloroplast transformation vector encoding a vitamin biosynthetic pathway.

Group XVIII, claim(s) 1, 51, 58 and 60, drawn to a chloroplast transformation vector encoding an antibiotic biosynthetic pathway.

Group XIX, claim(s) 1, 51, 58 and 60, drawn to a chloroplast transformation vector encoding a dye biosynthetic pathway.

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The inventions listed as Groups I-XIX do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Groups I-VIII and X-XIX are unrelated to Group IX. The groups do not share a technical feature. Groups I-VIII and X-XIX are drawn to chloroplast transformation vectors, not required by Group IX. Group IX, on the other hand, is drawn to a method of combatting insects via topical application of proteins, which are not required by Groups I-VIII and X-XIX.

Groups I-VIII and X-XIX do not relate to a single general inventive concept because these inventions do not constitute an advance over the prior art. The technical feature shared by the inventions is a chloroplast transformation vector encoding multi-gene operon. This technical feature is disclosed by Maliga et al (US 5,877,402 A, 02 March, 1999, see Figure 22A and column 65, lines 19-39), who teach chloroplast transformation vectors where more than one gene is expressed from the same promoter. Thus, the shared technical feature is not special. These groups are deemed to lack unity of invention because they are not so linked to form a single general inventive concept.

The following sets of Groups are unrelated to one another: Groups I-VI, Groups VII-VIII, Groups X-XI and XIII-XIX, and Group XII. Groups I-VI are drawn to chloroplast transformation vectors encoding Bt toxins, Groups VII-VIII are drawn to chloroplast transformation vectors encoding a biopharmaceutical protein, Groups X-XI and XIII-XIX are drawn to chloroplast transformation vectors encoding biosynthetic pathways, and Group XII is drawn to chloroplast transformation vectors encoding a monoclonal antibody. Thus the Groups do not share a special technical feature.

The technical feature shared by Groups I-VI is a chloroplast transformation vector encoding a Bt toxin. This technical feature is disclosed by McBride et al (US 5,545,818 A, 13 August, 1996, see Figure 1, column 2, lines 24-60, and column 7, line 63, to column 8, line 5), who teach chloroplast transformation vectors that encode Bacillus thuringiensis crystal proteins. Thus, the shared technical feature is not special, and these groups are deemed to lack unity of invention because they are not so linked to form a single general inventive concept. The Groups are unrelated to each other because the vectors of each encode an additional protein, and this additional protein differs among each Group.

The technical feature shared by Groups VII and VIII is a chloroplast transformation vector encoding biopharmaceutical protein. This technical feature is not special because it is disclosed by Maliga et al (US 5,877,402 A, 02 March, 1999, see column 27, lines 14-32), who suggest the expression of insulin in plant chloroplasts. These Groups are thus deemed to lack unity of invention because they are not so linked to form a single general inventive concept. Group VII is unrelated to Group VIII because Group VII vectors encode insulin, while Group VIII vectors encode human albumin.

The technical feature shared by Groups X-XI and XIII-XIX is a chloroplast transformation vector encoding a biosynthetic pathway. This technical feature is not special because it is disclosed by Maliga et al (US 5,877,402 A, 02 March, 1999, see column 27, lines 53-59), who suggest the expression of biosynthetic pathway genes in plastids. As the Groups are drawn to different biosynthetic pathways, the Groups are unrelated.

The technical feature shared by Groups X-XI is organisms that express both MerA and MerB. This technical feature is not special because it is disclosed by Meagher et al (US 5,965,796 A 12 October, 1999, see column 14, lines 2-8 and column 12, lines 8-36), who suggest the expression both genes in the same plant and the use of those plants in bioremediation. Group X is unrelated to Group XI because Group X is drawn to expression of those genes in higher plants, while Group XI is drawn to expression of those genes in single-cell photosynthetic organisms.

'ATENT COOPERATION TR[illegible] TY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark Office, PCT
2011 South Clark Place Room CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 28 January 2002 (28.01.02)	
International application No. PCT/US01/06276	**Applicant's or agent's file reference** 1464-PCT-00
International filing date (day/month/year) 28 February 2001 (28.02.01)	**Priority date** (day/month/year) 29 February 2000 (29.02.00)
Applicant DANIELL, Henry et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

21 September 2001 (21.09.01)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Denise POSPIEZNY
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
1464-PCT-00		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US01/06276	28 FEBRUARY 2001	29 FEBRUARY 2000
International Patent Classification (IPC) or national classification and IPC Please See Supplemental Sheet.		
Applicant AUBURN UNIVERSITY		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 15 sheets.

   ☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of ~~~ sheets.

3. This report contains indications relating to the following items:

   I ☒ Basis of the report

   II ☐ Priority

   III ☒ Non-establishment of report with regard to novelty, inventive step or industrial applicability

   IV ☒ Lack of unity of invention

   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

   VI ☐ Certain documents cited

   VII ☒ Certain defects in the international application

   VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
21 SEPTEMBER 2001	27 SEPTEMBER 2002
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer Jayne Badgero ANNE R. KUBELIK
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US01/06276

I. Basis of the report

1. With regard to the **elements** of the international application:*

☐ the international application as originally filed

☒ the description:
pages (See Attached) , as originally filed
pages _____ , filed with the demand
pages _____ , filed with the letter of _____

☒ the claims:
pages (See Attached) , as originally filed
pages _____ , as amended (together with any statement) under Article 19
pages _____ , filed with the demand
pages _____ , filed with the letter of _____

☒ the drawings:
pages (See Attached) , as originally filed
pages _____ , filed with the demand
pages _____ , filed with the letter of _____

☒ the sequence listing part of the description:
pages (See Attached) , as originally filed
pages _____ , filed with the demand
pages _____ , filed with the letter of _____

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:

☐ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).

☐ the language of publication of the international application (under Rule 48.3(b)).

☐ the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

☐ contained in the international application in printed form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ The statement that the information recorded in computer readable form is identical to the writen sequence listing has been furnished.

4. ☒ The amendments have resulted in the cancellation of:

☒ the description, pages NONE

☒ the claims, Nos. NONE

☒ the drawings, sheets/~~fig~~ NONE

5. ☐ This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

* *Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

***Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.*

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been and will not be examined in respect of:

☐ the entire international application.

☒ claims Nos. 7, 9, 11, 13, 33-36, 56-57

because:

☐ the said international application, or the said claim Nos. _ relate to the following subject matter which does not require international preliminary examination *(specify)*.

☐ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. _ are so unclear that no meaningful opinion could be formed *(specify)*.

☐ the claims, or said claims Nos. _ are so inadequately supported by the description that no meaningful opinion could be formed.

☒ no international search report has been established for said claims Nos. (See Attached).

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

- [ ] restricted the claims.
- [X] paid additional fees.
- [ ] paid additional fees under protest.
- [ ] neither restricted nor paid additional fees.

2. [ ] This Authority found that the requirement of unity of invention is not complied with and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

- [ ] complied with.
- [X] not complied with for the following reasons:

Please See Supplemental Sheet.

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

- [ ] all parts.
- [X] the parts relating to claims Nos. 1-6, 8, 10, 12, 14-32, 37-55, 58-60.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
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V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. statement

Novelty (N)	Claims	(Please See supplemental sheet)	YES
	Claims	(Please See supplemental sheet)	NO
Inventive Step (IS)	Claims	(Please See supplemental sheet)	YES
	Claims	(Please See supplemental sheet)	NO
Industrial Applicability (IA)	Claims	(Please See supplemental sheet)	YES
	Claims	(Please See supplemental sheet)	NO

2. citations and explanations (Rule 70.7)

Amended page 50, (claims 27-47) is not proper and has not been entered.

Claims 1-6, 8, 10, 12, 14-32, 37-55, and 58-60 meet the criteria set out in PCT Article 33 (4) because plastid transformation vectors, methods of transforming plastids and plants with transformed plastids have industrial applicability.

Claims 4-6, 10, 12, 15-20, 24-32, 39-42, 44-47, 49, 53-55 and 59 meet the criteria set out in PCT Article 33(2), because the prior art does not teach plastid transformation vectors encoding a Bt crystal protein and a chaperonin, or a Bt crystal protein and a non-Bt toxin, or insulin, nor does it teach plants whose plastids are transformed with those vectors. The prior art also does not teach eukaryotic plants whose plastids have been transformed with mercury resistance genes.

Claims 1, 51, 58 and 60 lack novelty under PCT Article 33(2) as being anticipated by MALIGA et al (US 5,877,402, 1999). Maliga et al teach a plastid transformation vector that comprises a promoter driving a multi-gene operon, a selectable marker sequence, a multi-gene operon encoding a protein, a transcription termination region, and flanking regions homologous to the target plastid genome. Maliga et al also teach methods of plastid transformation (Fig. 30, column 65, lines 19-39, and column 66, lines 16-39). Some of the plastid transformation vectors produce dye because they have the beta-galactosidase gene.

In the response filed 4 March, 2002,Applicant urges that Maliga et al does not teach a promoter driving a mutligene operon, but instead teaches fusing a promoterless uidA coding region with the rbcL 3'UTR and showed readthrough from the native rbcL 3'UTR to the exogenous uidA. Applicant urges that because the open reading frames are not expressed in a 1:1 ratio they do not constitute an operon. Applicant also urges (Continued on Supplemental Sheet.)

International application No.
PCT/US01/06276

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

Claims 1, 3 and 37 are objected to under PCT Rule 66.2(a)(iii) as containing the following defect(s) in the form or contents thereof:

Misspelling was noted of "biosynethtic" in claim 37.

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

The description is objected to under PCT Rule 66.2(a)(v) as lacking clarity under PCT Article 5 because it fails to adequately enable practice of the claimed invention because: cyanobacteria like Synechocytis do not have plastids, yet claim 50 requires that Synechocytis be transformed with a plastid transformation vector.

Claims 1, 21-23, 37-38, 43, 48 and 50 are objected to as lacking clarity under PCT Rule 66.2(a)(v) because practice of the claimed invention is not enabled as required under PCT Rule 5.1(a) for the reasons set forth in the immediately preceding paragraph.

The description is objected to under PCT Rule 66.2(a)(v) as lacking clarity under PCT Article 5 because it fails to contain an adequate written description of DNA sequences for homologous integration into the Synechocytis genome. The description is inadequate because such sequences are neither presented in the description nor cited in a reference.

Claims 1, 21-23, 37-38, 43, 48 and 50 are objected to as lacking clarity under PCT Rule 66.2(a)(v) because practice of the claimed invention is not adequately described in writing, as required under PCT Rule 5.1(a)(iii), for the reasons set forth in the immediately preceding paragraph.

Claims 12, 16, 18 and 54 are objected to under PCT Rule 66.2(a)(v) as lacking clarity under PCT Article 6 because the claims are indefinite for the following reason(s): Claim 14 is drawn to a method of controlling an insect by topical application of a protein, while dependent claim 16 is drawn to a method using a step of culturing plant cells. For purposes of examination, claim 16 was treated as though it were dependent upon claim 15, which is a method of transforming the chloroplast of a plant. Similarly, dependent claim 18 is drawn to a transformed plant of any one of claims 14-16. As claim 14 is not drawn to a transformed plant, for purposes of examination, claim 18 was treated as though it were dependent upon either claim 15 or 16.

Claim 12 recites the limitation "the putative chaperonin" in line 2. There is insufficient antecedent basis for this limitation in the claim.

Claim 21 recites the limitation "the biosynthetic pathway" in line 1. There is insufficient antecedent basis for this limitation in the claim.

(Continued on Supplemental Sheet.)

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Continuation of: Boxes I - VIII

CLASSIFICATION:
The International Patent Classification (IPC) and/or the National classification are as listed below:
IPC(7): A01H 5/02, 5/10, 13/00; C12N 1/20, 1/12, 5/04, 15/29, 15/32, 15/52, 15/31, 15/17, 15/13, 15/63, 15/74, 15/82 and
US Cl.: 435/320.1, 468, 252.3, 430, 418, 419; 536/23.71, 23.5, 23.6, 23.7, 23.2, 23.53; 800/278, 279, 288, 298, 302, 298, 287; 514/2

I. BASIS OF REPORT:

This report has been drawn on the basis of the description,
page(s) 1-45, as originally filed.
page(s) NONE, filed with the demand.
and additional amendments:
NONE

This report has been drawn on the basis of the claims,
page(s) 47-49, 50-51, as originally filed.
page(s) NONE, as amended under Article 19.
page(s) NONE, filed with the demand.
and additional amendments:
Page 46, filed with the letter of 04 March, 2002.

This report has been drawn on the basis of the drawings,
page(s) 1-14, as originally filed.
page(s) NONE, filed with the demand.
and additional amendments:
NONE

This report has been drawn on the basis of the sequence listing part of the description:
page(s) NONE, as originally filed.
pages(s) NONE, filed with the demand.
and additional amendments:
NONE

III. NON-ESTABLISHMENT OF REPORT:

No international search report has been established for claim numbers 7, 9, 11, 13, 33-36, 56-57.

IV. LACK OF UNITY OF INVENTION:

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2, and 13.3 is not complied with for the following reasons:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-3, 6, 15-20 and 60, drawn to a chloroplast transformation vector encoding a Bt toxin and a chaperonin.

Group II, claim(s) 1, 4-6, 55 and 60, drawn to a chloroplast transformation vector encoding a Bt toxin and a cholesterol oxidase.

Group III, claim(s) 1, 4-6, 55 and 60, drawn to a chloroplast transformation vector encoding a Bt toxin and an alpha-amylate inhibitor.

Group IV, claim(s) 1, 4-6, 55 and 60, drawn to a chloroplast transformation vector encoding a Bt toxin and a protease

Supplemental Box
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Continuation of: Boxes I - VIII

inhibitor.

Group V, claim(s) 1, 4-6, 55 and 60, drawn to a chloroplast transformation vector encoding a Bt toxin and a cowpea trypsin inhibitor.

Group VI, claim(s) 1, 4-6, 55 and 60, drawn to a chloroplast transformation vector encoding a Bt toxin and a potato proteinase inhibitor II.

Group VII, claim(s) 1, 8, 10, 12 and 59, drawn to a chloroplast transformation vector encoding insulin.

Group VIII, claim(s) 1, 8, 10, 12 and 59, drawn to a chloroplast transformation vector encoding human albumin.

Group IX, claim(s) 14, drawn to a method of combating insects via application of proteins.

Group X, claim(s)1, 21-32, 37-42 and 60, drawn to a plant chloroplast transformation vector comprising MerA and MerB, a method of transforming a chloroplast with that vector to confer resistance to metal ions, and a plant so obtained.

Group XI, claim(s) 1, 23 and 37-50, drawn to a chloroplast transformation vector comprising MerA and MerB for transformation of a single-celled organism, a method of transformation, a method of phytoremediation, and transformed algae and cyanobacteria.

Group XII, claim(s) 1, 51-54 and 60, drawn to a chloroplast transformation vector encoding a monoclonal antibody.

Group XIII, claim(s) 1, 58 and 60, drawn to a chloroplast transformation vector encoding an amino acid biosynthetic pathway.

Group XIV, claim(s) 1, 58 and 60, drawn to a chloroplast transformation vector encoding an fatty acid biosynthetic pathway.

Group XV, claim(s) 1, 58 and 60, drawn to a chloroplast transformation vector encoding a carbohydrate biosynthetic pathway.

Group XVI, claim(s) 1, 58 and 60, drawn to a chloroplast transformation vector encoding a polymer biosynthetic pathway.

Group XVII, claim(s) 1, 58 and 60, drawn to a chloroplast transformation vector encoding a vitamin biosynthetic pathway.

Group XVIII, claim(s) 1, 58 and 60, drawn to a chloroplast transformation vector encoding an antibiotic biosynthetic pathway.

Group XIX, claim(s) 1, 58 and 60, drawn to a chloroplast transformation vector encoding a dye biosynthetic pathway.

The inventions listed as Groups I-XIX do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Groups I-VIII and X-XIX are unrelated to Group IX. The groups do not share a technical feature. Groups I-VIII and X-XIX are drawn to chloroplast transformation vectors, not required by Group IX. Group IX, on the other hand, is drawn to a method of combating insects via topical application of proteins, which are not required by Groups I-VIII and X-XIX.

Groups I-VIII and X-XIX do not relate to a single general inventive concept because these inventions do not constitute an advance over the prior art. The technical feature shared by the inventions is a chloroplast transformation vector encoding multi-gene operon. This technical feature is disclosed by Maliga et al (US 5,877,402 A, 02 March, 1999, see Figure 22A and column 65, lines 19-39), who teach chloroplast transformation vectors where more than one gene is expressed from the same promoter. Thus, the shared technical feature is not special. These groups are deemed to lack unity of invention because they are not so linked to form a single general inventive concept.

The following sets of Groups are unrelated to one another: Groups I-VI, Groups VII-VIII, Groups X-XI and XIII-XIX, and Group XII. Groups I-VI are drawn to chloroplast transformation vectors encoding Bt toxins, Groups VII-VIII are drawn to chloroplast transformation vectors encoding a biopharmaceutical protein, Groups X-XI and XIII-XIX are drawn to chloroplast transformation vectors encoding biosynthetic pathways, and Group XII is drawn to chloroplast transformation vectors encoding a monoclonal antibody. Thus the Groups do not share a special technical feature.

The technical feature shared by Groups I-VI is a chloroplast transformation vector encoding a Bt toxin. This technical f eature

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Continuation of: Boxes I - VIII

is disclosed by McBride et al (US 5,545,818 A, 13 August, 1996, see Figure 1, column 2, lines 24-60, and column 7, line 63, to column 8, line 5), who teach chloroplast transformation vectors that encode Bacillus thuringiensis crystal proteins. Thus, the shared technical feature is not special, and these groups are deemed to lack unity of invention because they are not so linked to form a single general inventive concept. The Groups are unrelated to each other because the vectors of each encode an additional protein, and this additional protein differs among each Group.

The technical feature shared by Groups VII and VIII is a chloroplast transformation vector encoding biopharmaceutical protein. This technical feature is not special because it is disclosed by Maliga et al (US 5,877,402 A, 02 March, 1999, see column 27, lines 14-32), who suggest the expression of insulin in plant chloroplasts. These Groups are thus deemed to lack unity of invention because they are not so linked to form a single general inventive concept. Group VII is unrelated to Group VIII because Group VII vectors encode insulin, while Group VIII vectors encode human albumin.

The technical feature shared by Groups X-XI and XIII-XIX is a chloroplast transformation vector encoding a biosynthetic pathway. This technical feature is not special because it is disclosed by Maliga et al (US 5,877,402 A, 02 March, 1999, see column 27, lines 53-59), who suggest the expression of biosynthetic pathway genes in plastids. As the Groups are drawn to different biosynthetic pathways, the Groups are unrelated.

The technical feature shared by Groups X-XI is organisms that express both MerA and MerB. This technical feature is not special because it is disclosed by Meagher et al (US 5,965,796 A 12 October, 1999, see column 14, lines 2-8 and column 12, lines 8-36), who suggest the expression both genes in the same plant and the use of those plants in bioremediation. Group X is unrelated to Group XI because Group X is drawn to expression of those genes in higher plants, while Group XI is drawn to expression of those genes in single-cell photosynthetic organisms.

V. 1. REASONED STATEMENTS:
The report as to Novelty was positive (YES) with respect to claims 4-6, 10, 12, 15-20, 24-32, 39-42, 44-47, 49, 53-55 and 59.
The report as to Novelty was negative (NO) with respect to claims 1-3, 8, 14, 21-23, 37-38, 43, 48, 50-52, 58 and 60.
The report as to Inventive Step was positive (YES) with respect to claims NONE.
The report as to Inventive Step was negative (NO) with respect to claims 1-6, 8, 10, 12, 14-32, 37-55 and 58-60.
The report as to Industrial Applicability was positive (YES) with respect to claims 1-6, 8, 10, 12, 14-32, 37-55 and 58- 60.
The report as to Industrial Applicability was negative (NO) with respect to claims NONE.

V. 2. REASONED STATEMENTS - CITATIONS AND EXPLANATIONS (Continued):
that Maliga et al teaches introduction of only a single foreign gene. To support the uniqueness of the instant invention, Applicant introduces an in-press article in which the instant inventor is one of the authors.

This is not found persuasive because Maliga et al teach constructs comprising uidA and aadA and that are expressed behind a single promoter (Figs 22A and 25A). Maliga et al also suggest expressing multople genes behind a single promoter (column 65, lines 19-39). Neither the specification nor the claims define an operon as a polycistron in which the open reading frames are expressed in a 1:1 ratio; thus, Applicant is arguing limitations not found in the claims.

Claims 1-3, 14 and 60 lack novelty under PCT Article 33(2) as being anticipated by MCBRIDE et al (US 5,545,817, 1996) .

McBride et al teach plastid transformation vectors that encode Bacillus thuringiensis (Bt) crystal proteins (column 7, line 64, to column 8, line 67). McBride et al also teach a method of controlling insects by the application of a crystal protein to their environment (column 10, lines 45, to column 11, line 13). McBride et al state that the plastid transformation constructs of their invention may include a "number of consecutive encoding regions, to be expressed as an operon, for example where introduction of a foreign biochemical pathway into plastids is desired" (column 2, lines 56-63).

Claims 1-3, 8, 51-52 and 60 lack novelty under PCT Article 33(2) as being anticipated by CALGENE LLC (WO 00/03012).

Calgene LLC teaches plastid transformation vectors encoding the biopharmaceutical proteins aprotinin and human growth hormone (pages 8-9, 23-24, and 37-40) and ones encoding Bacillus thuringiensis (Bt) crystal proteins (pg 21).

Claims 1, 51, 58 and 60 lack novelty under PCT Article 33(2) as being anticipated by KHAN et al (1999, Nature Biotechnol. 17:910-915).

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Continuation of: Boxes I - VIII

Khan et al teach plastid transformation vectors encoding a fusion protein between a green fluorescent protein and an antibiotic resistance protein (entire article).

Claims 1-3, 14, 51, and 60 lack novelty under PCT Article 33(2) as being anticipated by KOTA et al (1999, Proc. Natl. Acad. Sci. USA 96:1840-1845).

Kota et al teach a plastid transformation vector encoding a Bacillus thuringiensis (Bt) crystal protein (pg 1842). Kota et al also teach a method of controlling insects by the application of a crystal protein to their environment (pg 1843).

Claims 1, 51, 58 and 60 lack novelty under PCT Article 33(2) as being anticipated by DANIELL et al (US 6,004,782, 1999).

Daniell et al teach plastid transformation vectors encoding the polymer poly(VPGVP) (column 20, line 31, to column 21, line 6).

Claim 14 lacks novelty under PCT Article 33(2) as being anticipated by FUCHS et al (US 5,250,515, 1993).

Fuchs et al teach a method of controlling insects by the application of a crystal protein to their environment (column 28, lines 19-31, and claims 1-4).

Claims 1-4, 51 and 60 lack an inventive step under PCT Article 33(3) as being obvious over TURKEC (1999, Turk. J. Field Crops 4:85-90) in view of each of BAUMANN et al (1988, J. Bacteriol. 170:2045-2050) and CRICKMORE et al (1998, Microbiol. Mol. Biol. Rev. 62:807-813).

The claims are drawn to plastid transformation vectors encoding more than one Bt crystal protein in the form of an operon.

Turkec teaches plastid transformation vectors that encode two Bacillus sphaericus crystal toxin proteins expressed behind a single promoter. Turkec does not teach plastid transformation vectors encoding more than one Bt crystal protein expressed behind a single promoter.

Baumann et al teach that the genes used by Turkec are expressed in B. sphaericus within a single transcrirtional operon (pg 2047, right column).

Crickmore et al teach a variety of Bt crystal protein genes.

At the time the invention was made, it would have been obvious to one of ordinary skill in the art to modify the plastid transformation vectors encoding B. sphaericus crystal proteins as taught by Turkec, and to express Bt crystal protein genes as described in Crickmore et al. One of ordinary skill in the art would have been motivated to do so because of the suggestion of Turkec to do so (pg 85 and 88).

Claims 1-3, 6, 15-20 and 60 lack an inventive step under PCT Article 33(3) as being obvious over KOTA et al (1999, Proc. Natl. Acad. Sci. USA 96:1840-1845) in view of Daniell et al (1994, NATO ASI Series, Vol H 86, Biochemical and Cellular Mechanism of Stress Tolerance in Plants, Cherry, ed. Springer-Verlag Berlin, pages 589-604).

The claims are drawn to a plastid transformation vector comprising an expression cassette comprising in a 5' to 3' direction of translation a plastid promoter, a selectable marker coding sequence, an operon, and a transcription termination sequence and that have flanking sequences, wherein the operon encodes an insecticidal toxin crystal protein and a protein involved in folding the toxin.

Kota et al teach a plastid transformation vector comprising an expression cassette comprising the aadA coding sequence, which provides resistance to spectinomycin, and the *cry*2Aa2 *B. thuringiensis* crystal protein coding sequence expressed behind the plastid promoter *Prrn* and flanking seqùences rbcL and accD (pg 1842, left column, paragraph 1, and Figure 1). Kota et al also teach a plastid transformation method comprising transformation of plant cells with the vector, growing the transformed plant cells under conditions that would allow expression of the coding region of the vector, selection of the plant cells in a medium comprising spectinomycin, and regeneration of the selected cells into a plant (pg 1841, left column, paragraph 2, and the paragraph spanning pg 1841-1842). Kota et al do not disclose such vectors comprising a chaperonin coding sequence in the expression cassette or a *cry*2Aa2 operon.

Daniell et al teach the expression of the full-length cryIIA operon in *E. coli* which encodes a chaperonin (pg 595, paragraph 1), and suggest expressing the entire operon in tobacco chloroplasts (pg 597, paragraph 3). cryIIA is an earlier name for *cry*2Aa2.

At the time the invention was made, it would have been obvious to one of ordinary skill in the art to modify the method of plastid transformation with the *cry*2Aa2 coding sequence as taught by Kota et, to express the Bt operon as described in Daniell et al. One of ordinary skill in the art would have been motivated to do so because of the suggestion of Kota et al to express the entire *cry*2Aa2 operon in chloroplasts because ORFs required for folding Cry2Aa2 proteins are present in that operon (pg 1844, left column, paragraph 1) and because of the suggestion of Daniell et al to express the entire operon in tobacco chloroplasts (pg 597, paragraph 3).

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Continuation of: Boxes I - VIII

Claims 1-4, 14, 51 and 60 lack an inventive step under PCT Article 33(3) as being obvious over each of MCBRIDE et al (US 5,545,818, 1996) and KOTA et al (1999, Proc. Natl. Acad. Sci. USA 96:1840-1845). in view of CRICKMORE et al (1998, Microbiol. Mol. Biol. Rev. 62:807-813).

The claims are drawn to plastid transformation vectors encoding a variety of Bt crystal protein.

McBride et al teach plastid transformation vectors that encode Bt crystal proteins and methods of plastid transformation (column 6, lines 3-15, and column 7, line 53, to column 8, line 5). McBride et al do not disclose a variety of Bt crystal protein genes.

Kota et al teach a plastid transformation vector encoding a Bt crystal protein and methods of plastid transformation (pg 1842). Kota et al do not teach a variety of Bt crystal protein genes.

Crickmore et al teach a variety of Bt crystal protein genes.

At the time the invention was made, it would have been obvious to one of ordinary skill in the art to produce plants transformed with plastid transformation vectors encoding a Bt crystal protein as taught by each of McBride et al and Kota et al, and to modify that to express other or multiple Bt crystal protein genes as described in Crickmore et al. One of ordinary skill in the art would have been motivated to do so because of the advantages of plastid expression of Bt crystal proteins (Kota et al pg 1844).

Claims 1-6, 14-20, 51 and 60 lack an inventive step under PCT Article 33(3) as being obvious over the prior art as applied in the immediately preceding paragraph and further in view of BOSTON et al (1996, Plant Mol. Bio. 32:191-222).

The claims are drawn to plastid transformation vectors encoding a Bt crystal protein and a chaperonin, methods of plastid transformation with those vectors and plants transformed with them.

Each of McBride et al and Kota et al in view of Crickmore et al disclose plastid transformation vectors encoding a variety of Bt crystal protein genes and plants transformed with those vectors. Each of McBride et al and Kota et al in view of Crickmore et al do not disclose plastid transformation vectors encoding a Bt crystal protein and a chaperonin.

Boston et al teach the importance of chaperonins in plastid protein folding and that the gene for chaperonin is plastid-encoded in some plants (pg 204-207 and 209).

At the time the invention was made, it would have been obvious to one of ordinary skill in the art to produce plants transformed with plastid transformation vectors encoding a variety of Bt crystal protein genes as taught by each of McBride et al and Kota et al in view of Crickmore et al, and to modify that to also express chaperonins in those constructs as described in Boston et al. One of ordinary skill in the art would have been motivated to do so because of the importance of chaperonins in plastid gene expression (Boston et al, pg 204-207).

Claims 1, 51, 58 and 60 lack an inventive step under PCT Article 33(3) as being obvious over MALIGA et al (US 5,877,402, 1999) in view of GRUYS et al (US 5,958,745, 1999).

The claims are drawn to plastid transformation vectors encoding an amino acid biosynthetic pathway.

Maliga et al disclose teach a plastid transformation vector that comprises a promoter driving a multi-gene operon, a selectable marker sequence, a multi-gene operon encoding a protein, a transcription termination region and flanking regions homologous to the target plastid genome and methods of plastid transformation (Fig. 30, column 65, lines 19-39, and column 66, lines 16-39). Maliga et al suggest putting gene clusters encoding complex biosynthetic pathways into plastid transformation vectors (column 27, lines 14-59; column 65, lines 28-39). Maliga et al do not teach genes encoding those pathways.

Gruys et al teach plants transformed with genes of the E. coli isoleucine biosynthetic pathway; these genes are targeted to the chloroplast via transit peptides (column 69, line 9, to column 72, line 18).

At the time the invention was made, it would have been obvious to one of ordinary skill in the art to transform plant plastids as taught by Maliga et al, and to modify that to use genes encoding amino acid biosynthesis proteins as described in Gruys et al. One of ordinary skill in the art would have been motivated to do so because of the suggestion of Maliga et al to do so (column 65, lines 28-39) and because selection of transformation of the chloroplast by transformation with a plastid transformation vector versus transformation using chloroplast transit peptides is an obvious design choice.

Claims 1, 51, 58 and 60 lack an inventive step under PCT Article 33(3) as being obvious over MALIGA et al (US 5,877,402, 1999) in view of PATTON (US 5,869,719, 1999).

The claims are drawn to plastid transformation vectors encoding a vitamin biosynthetic pathway.

Maliga et al disclose teach a plastid transformation vector that comprises a promoter driving a multi-gene operon, a selectable marker sequence, a multi-gene operon encoding a protein, a transcription termination region and flanking regions homologous to the target plastid genome and methods of plastid transformation (Fig. 30, column 65, lines 19-39, and column 66, lines 16-39). Maliga et al suggest putting gene clusters encoding complex biosynthetic pathways into plastid transformation vectors (column 27, lines 14-59; column 65, lines 28-39). Maliga et al do not teach genes encoding those pathways.

Patton teaches plants transformed with biotin biosynthetic genes (column 22, line 5, to column 24, line 25).

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(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

At the time the invention was made, it would have been obvious to one of ordinary skill in the art to transform plant plastids as taught by Maliga et al, and to modify that to use genes encoding amino acid biosynthesis proteins as described in Patton. One of ordinary skill in the art would have been motivated to do so because of the suggestion of Maliga et al to do so (column 65, lines 28-39), because of the suggestion of Patton to do so (column 9, lines 37-44), and because selection of transformation of the chloroplast by transformation with a plastid transformation vector versus transformation using chloroplast transit peptides is an obvious design choice.

Claims 1, 8, 10, 12, 51-52 and 60 lack an inventive step under PCT Article 33(3) as being obvious over CALGENE LLC (WO 00/03012) in view of CARRINGTON et al (US 5,766,885, 1998).

The claims are drawn to plastid transformation vectors encoding insulin.

Calgene LLC teaches plastid transformation vectors encoding the biopharmaceutical proteins aprotinin and human growth hormone (pages 8-9, 23-24, and 37-40). Calgene LLC suggest expressing an insulin gene in plant plastids (pg 8, lines 31- 33), but do not disclose an insulin gene.

Carrington et al teach plants transformed with a gene encoding human insulin (column 10, lines 53-67, column 20, lines 30-36, and claim 8).

At the time the invention was made, it would have been obvious to one of ordinary skill in the art to express biopharmaceutical proteins in plant plastids as taught by Calgene LLC, and to modify that to make insulin that protein as described in Carrington et al. One of ordinary skill in the art would have been motivated to do so because of the suggestion of Calgene LLC to do so (pg 8, lines 31-33).

Claims 1, 8, 51-54 and 60 lack an inventive step under PCT Article 33(3) as being obvious over CALGENE LLC (WO 00/03012) in view of JOHN INNES CENTRE (WO 99/66026).

The claims are drawn to plastid transformation vectors encoding antibodies.

Calgene LLC teaches plastid transformation vectors encoding the biopharmaceutical proteins aprotinin and human growth hormone (pages 8-9, 23-24, and 37-40). Calgene LLC suggest expressing monoclonal antibodies in plant plastids (claims 11 and 21), but do not disclose an antibody gene.

John Innes Centre teaches plants transformed with genes encoding antibodies (pg 23-56). These antibodies would be monoclonal.

At the time the invention was made, it would have been obvious to one of ordinary skill in the art to express biopharmaceutical proteins in plant plastids as taught by Calgene LLC, and to modify that to make an antibody as that protein as described in John Innes Centre. One of ordinary skill in the art would have been motivated to do so because the suggestion of Calgene LLC to do so (claims 11 and 21) and because John Innes Centre suggests targeting the expression of the antibody gene to the chloroplast (pg 8).

Claims 1, 8, 51-52 and 59-60 lack an inventive step under PCT Article 33(3) as being obvious over CALGENE LLC (WO 00/03012) in view of SIJMONS et al (1990, Biotechnology 8:217-221).

The claims are drawn to plastid transformation vectors encoding human serum albumin.

Calgene LLC teaches plastid transformation vectors encoding the biopharmaceutical proteins aprotinin and human growth hormone (pages 8-9, 23-24, and 37-40). Calgene LLC do not disclose plastid transformation vectors encoding human serum albumin.

Sijmons et al teach the production of correctly processed human serum albumin in transgenic plants (pg 217-218).

At the time the invention was made, it would have been obvious to one of ordinary skill in the art to express biopharmaceutical proteins in plant plastids as taught by Calgene LLC, and to modify that to express human serum albumin as described in Sijmons et al. One of ordinary skill in the art would have been motivated to do so because the substitution of one biopharmaceutical protein for another is an obvious design choice.

Claims 1-5, 51, 55 and 60 lack an inventive step under PCT Article 33(3) as being obvious over MCBRIDE et al (US 5,545,818 A, 1996) in view of GREENPLATE et al (US 5,763,245, 1998).

The claims are drawn to vectors for transformation of a plant plastid with both a cholesterol oxidase gene and a B. thuringiensis crystal protein gene.

McBride et al teach plastid transformation vectors that encode B. thuringiensis crystal proteins (column 6, lines 3-15, and column 7, line 53, to column 8, line 5). McBride et al also teach a method of controlling insects by the application of a crystal protein to their environment (column 10, line 1-60). McBride et al do not disclose transformation of a plant with both a cholesterol oxidase gene and a B. thuringiensis crystal protein gene.

Greenplate et al teach plants transformed with a cholesterol oxidase gene in combination with a B. thuringiensis crystal protein gene (column 3, lines 3-9, and claims 11-18). Greenplate et al also suggest expression of these genes in the chloroplast (column 18, line 48-63).

At the time the invention was made, it would have been obvious to one of ordinary skill in the art to transform plastid with

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Continuation of: Boxes I - VIII

a vector encoding a B. thuringiensis crystal protein gene as taught by McBride et al, and to modify that to add the cholesterol oxidase gene as described in Greenplate et al. One of ordinary skill in the art would have been motivated to do so because of the suggestion of Greenplate et al to express these genes in the chloroplast (column 18, line 48-63).

Claims 1-5, 51, 55 and 60 lack an inventive step under PCT Article 33(3) as being obvious over the prior art as applied in the immediately preceding paragraph and further in view of each of CHEN et al (US 5,981,722, 1999) and THOMAS et al (US 5,436,392, 1995).

The claims are drawn to plastid transformation encoding both a proteinase inhibitor gene and a B. thuringiensis crystal protein gene.

McBride et al in view of Greenplate et al disclose plastid transformation encoding both a cholesterol oxidase gene and a B. thuringiensis crystal protein gene. McBride et al in view of Greenplate et al do not disclose vectors with proteinase inhibitor gene and a B. thuringiensis crystal protein gene.

Chen et al teach plants transformed with a trypsin inhibitor gene (claims 1-21).

Thomas et al teach plants transformed with a proteinase inhibitor gene (claims 1-16).

At the time the invention was made, it would have been obvious to one of ordinary skill in the art to transform plastids with vectors comprising two insect toxin genes as taught by McBride et al in view of Greenplate et al, and to modify that to use a proteinase inhibitor as one of those toxin genes as described in each of Chen et al and Thomas et al. One of ordinary skill in the art would have been motivated to do so because substitution of one insect toxin gene for another is an obvious design choice.

Claims 1, 21-32, 37-47, 51 and 60 lack an inventive step under PCT Article 33(3) as being obvious over MALIGA et al (US 5,877,402, 1999) in view of MEAGHER et al (US 5,965,796, 1999).

The claims are drawn to plastid transformation vectors encoding MerA and MerB, plants and seeds transformed with that vector, and methods of plastid transformation.

Maliga et al teach a plastid transformation vector that comprises a promoter driving a multi-gene operon, a selectable marker sequence, a multi-gene operon encoding a protein, a transcription termination region, and flanking regions homologous to the target plastid genome. Maliga et al also teach methods of plastid transformation (Fig. 30, column 65, lines 19-39, and column 66, lines 16-39). Maliga et al suggest putting gene clusters encoding complex biosynthetic pathways into plastid transformation vectors (column 27, lines 14-59; column 65, lines 28-39). Maliga et al do not disclose genes encoding MerA or MerB.

Meagher et al teach plants and their seeds transformed with genes encoding MerA and MerB (column 3, lines 31-45; column 3, line 58, to column 4, line 20, column 21, line 50, to column 25, line 53).

At the time the invention was made, it would have been obvious to one of ordinary skill in the art to transform plant plastids with multiple genes as taught by Maliga et al, and to modify that to express MerA and MerB as described in Meagher et al. One of ordinary skill in the art would have been motivated to do so because of the suggestion of Maliga et al to express a wide variety of genes in plastids (column 27, lines 14-59; column 65, lines 28-39).

Claims 1, 21-23, 37-38, 43, 48 and 50 lack novelty under PCT Article 33(2) as being anticipated by ROSSEN (US 5,571,722, 1996).

Rossen teaches photosynthetic bacteria transformed with the MerA and MerB genes (column 15, line 20, to column 18, line 42).

Claims 1, 21-23, 37-38, 43, 48-51 and 60 lack an inventive step under PCT Article 33(3) as being obvious over DUNAHAY et al (US 5,661,017, 1997) in view of ROSSEN (US 5,571,722, 1996).

The claims are drawn to Chlorella whose plastids have been transformed with genes encoding MerA and MerB.

Dunahay et al teach the transformation of the plastids of a variety of algae (claims 1-2 and 21) and suggest their use in bioremediation (column 1, lines 22-33). Dunahay et al do not disclose transformation of algal plastids with genes encoding MerA and MerB.

Rossen teaches photosynthetic bacteria transformed with the MerA and MerB genes and methods of phytoremediation (column 15, line 20, to column 18, line 42).

At the time the invention was made, it would have been obvious to one of ordinary skill in the art to transform algae plastids as taught by Dunahay et al, and to modify that to express MerA and MerB as described in Rossen. One of ordinary skill in the art would have been motivated to do so because of the overall utility in using single-celled photosynthetic organisms in bioremediation.

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

---------- NEW CITATIONS ----------

SIJMONS et al. Production of correctly processed human serum albumin in transgenic plants. Biotechnology. March 1990, Vol. 8, No. 3, pages 217-221, see pages 217-218.

US 5,545,817 A (MCBRIDE et al) 13 AUGUST 1996, see column 2, lines 56-63; column 7, line 64, to column 8, line 67; and column 10, line 45, to column 11, line 13

BAUMANN et al. Sequence Analysis of the Mosquitocidal Toxin Genes Encoding 51.4- and 41.9-Kilodalton Proteins from Bacillus sphaericus 2362 and 2297. J. Bacteriol. May 1988, Vol. 170, No. 5, pages 2045-2050. See Fig. 2 and pg 2047, right column.

TURKEC, Aydin. Chloroplast Transformation of Mosquitocidal Bacillus sphaericus Binary Toxin Genes in Chlamydomonas reinhardtii. Turk. J. Field Crops. 1999. Vol. 4, pages 85-90, see entire document.

DANIELL et al. Engineering plants for stress tolerance via organelle genomes. NATO ASI Series, Vol H 86, Biochemical and Cellular Mechanisms of STress Tolerance in Plants, ed. by J.H. Cherry, 1994, pages 589-604, see pages 595 and 597.

VIII. CERTAIN OBSERVATIONS ON THE APPLICATION (Continued):

In claim 54 it is not clear what the protein is in the same stoichiometric ratio to.